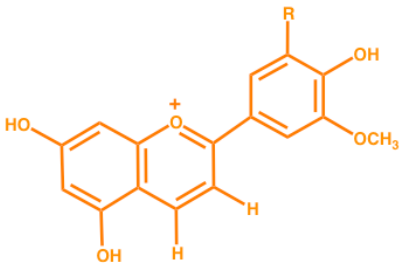

Deoxyanthocyanidins and derivatives: physical-chemical and antioxidant properties in aqueous solution

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2015

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“Only those who attempt the absurd can achieve the impossible.” – Albert Einstein

“Stay hungry. Stay foolish.” – Steve Jobs

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Resumo

Os polifenóis assumem um papel importante em matrizes alimentares, nomeadamente em vinhos, contribuindo directamente para as propriedades organolépticas, como a cor e o sabor. Alguns destes compostos, como as antocianinas e seus derivados apresentam cores intensas e são relativamente estáveis em solução aquosa. Esta característica confere a estes compostos a potencialidade de serem usados como corantes alimentares comerciais, o que tem conduzido consequentemente a avanços na pesquisa desenvolvida sobre a sua química ao longo dos anos.

O objectivo deste projecto foi de investigar e estudar pigmentos com características de cor e estabilidade interessantes em solução aquosa que possam ter potencial aplicação na indústria alimentar e de saúde, entre outras.

A estrutura química da forma de chalconas das antocianinas comuns é considerada muito importante em reações que conduzem à sua degradação irreversível, particularmente em soluções ácidas ou básicas fracas. No entanto, as desoxiantocianidas naturais são muito mais estáveis neste tipo de soluções do que as antocianinas e antocianidinas, conferindo-lhes a vantagem de serem usadas como corantes alimentares comerciais viáveis, justificando a investigação desenvolvida sobre a sua química, e em particular, a pesquisa por novos corantes com grande estabilidade. Além disso, outros estudos têm demonstrado outras potenciais aplicações para estes compostos, como o seu uso em tintas para cabelo, lasers, sensores de células fotovoltaicas e sistemas de memória molecular.

Sendo assim, vários pigmentos da família das desoxiantocianidinas foram sintetizados, isolados e estruturalmente caracterizados.

No primeiro trabalho foi estudada a reação entre procianidinas diméricas, que estão presentes em vinhos, e aldeídos cinâmicos que são extraídos de barricas de madeira durante o envelhecimento do vinho. Foi observada e confirmada a formação de novos pigmentos de cor laranja com uma estrutura em forma de aducto de oaklina-catequina. Estes aductos são classificados de desoxiantocianidinas, uma vez que não apresentam substituintes no carbono em posição *meta* comparativamente ao oxigénio com carga positiva do núcleo flavânico. O mecanismo hipotético de formação destes novos compostos foi elucidado e o aducto formado foi caracterizado estruturalmente por espectrometria de massa e RMN.

Foi descrita uma nova e simples síntese para obter 3-desoxiantocianidinas, nomeadamente a desoxipeonidina e a desoximalvidina, a partir da reação entre o floroglucinol e aldeídos cinâmicos (coniferaldeído e sinapaldeído).

A estabilidade de diferentes tipos de desoxiantocianidinas foi estudada por espectroscopia UV-Vis, comparando o seu comportamento e as suas propriedades fisico-químicas sob diferentes condições de pH e estímulo à luz com compostos análogos, como antocianinas comuns e outros flavílios.

Foram calculadas as constantes cinéticas e de equilíbrio da rede de reações químicas dependentes do pH e a sua fotoquímica foi avaliada.

Foi realizada a síntese de novos compostos análogos às piranoantocianinas (vitisinas). Os produtos resultantes foram designados de desoxivitisinas. A estabilidade da cor e a rede de reações químicas que ocorrem em solução aquosa após alterações de pH para estes compostos foi investigada aprofundadamente. As desoxivitisinas revelaram-se menos ácidas, e por conseguinte mais estáveis, que as correspondentes piranoantocianinas 3-O-glucosiladas.

Com um conhecimento profundo das propriedades das desoxiantocianidinas que advieram dos trabalhos anteriores, foi investigada a contribuição que outros efeitos poderiam ter nas propriedades da cor destes compostos. Assim, foram estudadas interações de copigmentação entre oaklinas (desoxiantocianidinas substituídas no anel A), que foram previamente identificadas em vinhos, com diversos copigmentos: catequina, epicatequina, ácido clorogénico, epigalhocatequina e a procianidina B3. Os resultados mostraram que as oaklinas, tal como as antocianinas, também apresentam interações de copigmentação que estabilizam ainda mais o catião flavílio em soluções hidroalcoólicas. Também foram realizadas simulações de dinâmica molecular para interpretar as ligações/interações químicas, especificando a posição relativa das moléculas do pigmento e copigmento nos complexos, e para interpretar as propriedades de absorção no visível dos complexos formados.

Por último, foram avaliadas as propriedades antioxidantes e antiproliferativas de várias desoxiantocianidinas através de métodos como o DPPH, FRAP, inibição da peroxidação de lipossomas e efeitos antiproliferativos em linhas celulares cancerígenas do estômago e do cólon. Os resultados mostraram que as desoxiantocianidinas apresentam propriedades antiradicalares, antioxidantes e antiproliferativas. Foi interessante observar que uma das desoxiantocianidinas, a oaklina guaiacilcatequinapirílio, apesar de apresentar menor capacidade antioxidante/antiradicalar nos ensaios de DPPH e FRAP quando comparada com a antocianina cianidina-3-glucósido, demonstrou um superior efeito antioxidante no modelo de lipossomas. O uso de lipossomas constitui um método promissor para avaliar propriedades antioxidantes relevantes para a nutrição humana, uma vez que usa uma membrana biológica. As oaklinas, que em estudos anteriores demonstraram ter propriedades interessantes quanto à sua cor e o seu uso em corantes

alimentares, devido à sua maior estabilidade em soluções de pH ácidas/neutras, também revelaram propriedades antioxidantes semelhantes às antocianinas comuns.
Este projeto permitiu um avanço científico significativo no estudo de um tipo de compostos derivados de antocianidinas, designados por desoxiantocianidinas. Estes compostos foram previamente identificados e descritos, mas nunca houve um profundo e extensivo estudo sobre as suas propriedades.

PALAVRAS-CHAVE: antocianinas, desoxiantocianidinas, oaklinas, corantes alimentares, envelhecimento do vinho, rede de reações flavílio, fotoquímica, copigmentos, propriedades antioxidantes, espectroscopia UV-Vis, espectrometria de massa, RMN

Abstract

Polyphenols assume an important role in food matrixes and namely in wines, contributing directly to their organoleptic properties such as color and flavor. Some of these compounds, such as anthocyanins and derivatives present intense colors and are relatively stable in aqueous solutions. This feature has given them the potential advantage to act as viable commercial food colorants, which has consequently led to advances in the research developed in their chemistry over the years.

The purpose of this project was to investigate and study pigments with interesting color features and stability in aqueous solutions that could have potential applications in food, health and other industries.

Open chalcone forms of the common anthocyanins are assumed to be crucial in reactions leading to irreversible degradation of anthocyanins, particularly under weakly acidic to weakly alkaline solution conditions. However, the natural deoxyanthocyanidins are much more stable in slightly acidic solutions than anthocyanins and anthocyanidins, which points to the potential advantage of this type of compounds as viable commercial food colorants, and justifies the research developed in the chemistry of 3-deoxyanthocyanins and, in particular, the search for new colorants with significant stability. In addition, more studies have demonstrated other potential applications for these compounds, such as their use as hair dyes, laser dyes, sensitizers for solar cells, and molecular-level memory systems.

Bearing this, several new pigments of the family of deoxyanthocyanidins were synthesized, isolated and structurally characterized.

In the first work, the reaction between dimeric procyanidins, which are present in real wines, and cinnamic aldehydes which are extracted from wood barrels during wine aging was studied. The formation of new orange pigments that were oaklin-catechin adducts was observed and confirmed. These adducts are classified as deoxyanthocyanidins, since they are not substituted in the carbon in *m*-position to the positively charged oxygen of the flavylium core. The hypothetic mechanism of formation of these new pigments was elucidated and the adduct formed was structurally characterized by mass spectrometry and NMR.

A new and simple synthesis procedure to obtain 3-deoxyanthocyanidins, particularly deoxypeonidin and deoxymalvidin, from the reaction between phloroglucinol and cinnamic aldehydes (coniferaldehyde and sinapaldehyde) was described.

The stability of different types of deoxyanthocyanidins was investigated by UV-Vis spectroscopy, comparing their behaviour and physical-chemical properties under

different pH conditions and light stimulus with analogous compounds such as common anthocyanins and other flavyliums.

The rate and equilibrium constants of the respective pH dependent network of chemical reactions presented by these compounds were calculated and their photochemistry evaluated.

The synthesis of new compounds analogous to pyanoanthocyanins (visitins) was carried out. The resulting products were designated deoxyvitisins. The color stability and the network of chemical reactions occurring in aqueous solution upon pH variations for the newly synthesized deoxyvitisins were thourougly investigated. Deoxyvitisins were found to be less acidic, thus more stable, than the corresponding pyranoanthocyanin 3-O-glucosides.

With a deeper understanding of the physical-chemical properties of deoxyanthocyanidins provided by the previous works, it was investigated the contribution that other effects might have on color features of these compounds. Hence, copigmentation interactions were studied between oaklins (ring A substituted deoxyanthocyanidins), which have been previously identified in wines, and several copigments: catechin, epicatechin, chlorogenic acid, epigallocatechin, and procyanidin B3. The results showed that oaklins, like common anthocyanins, also present copigmentation interactions that further stabilize the flavylium cation in hydroalcoholic solutions. Molecular dynamic simulations were also performed to interpret the binding data, to specify the relative arrangement of the pigment and copigment molecules within the complexes, and to interpret their absorption properties in the visible range.

Finally, the antioxidant and antiproliferative properties of several deoxyanthocyanidins were evaluated by DPPH, FRAP, liposomes peroxidation inhibiton assays and antiproliferative effects against cancer cell lines from stomach and colon. The results showed that all the deoxyanthocyanidins tested demonstrated antiradicalar, antioxidant and antiproliferative properties. It was very interesting to observe that one of the deoxyanthocyanidins, the oaklin guaiacylcatechinpyrylium, despite showing lower antioxidant/antiradicalar capacity in DPPH and FRAP assays when compared to an anthocyanin (cyanidin-3-glucoside), revealed a surprising higher antioxidant effect in the liposomes model. The use of liposomes constitutes a more promising method for assessing antioxidant properties relevant to human nutrition, since it uses a model biological membrane. Oaklins, which were previously shown to have interesting color features for its use as food colorants, due to their higher color stability in acidic/neutral pH solutions, also revealed antioxidant properties similar to common anthocyanins.

This research has allowed a significative scientific advance in the study of a specific anthocyanidin derived type of coumpounds, named deoxyanthocyanidins. They have been previously identified and described but there has never been a profound and extensive study about their characteristics.

KEYWORDS: anthocyanins, deoxyanthocyanidins, oaklins, food colorants, wine aging, flavylium network reactions, photochemistry, copigments, antioxidante properties, UV-Vis spectroscopy, mass spectrometry, NMR

Table of Contents

List of Figures

List of Tables

List of Abbreviations and Symbols

Abs – Absorbance
ACN – Acetronitrile
Api - Apigeninidin
DMSO/TFA – Dimethylsulfoxide/Trifluoroacetic acid
Con – Coniferaldehyde
COSY – Correlation Spectroscopy
Cy-glc – cyanidin-3-glucoside
d – Duplet
Cy – Cyanidin
Dp – Delphinidin
DPPH – 2,2-Diphenyl-1-picrylhydrazyl
DAD – Diode Array Detector
Dom - deoxymalvidin
Dop – deoxypeonidin
ESI – ElectroSpray Inonization
FRAP – Ferric Reducing Antioxidant Power
Fur – Furfural
Gcp - guaiacylcatechinpyrylium
Glc – Glucose
HMBC – Heteronuclear Multiple Bond Correlation
HPLC – High Performance Liquid Chromatography
HSQC – Heteronuclear Single Quantum Correlation
^{1}H – Proton NMR spectrum
J – Coupling constant
LC – Liquid Chromatography
Lut - Luteolinidin
MeOH – Methanol
min – Minutes
MS – Mass Spectrometry
Mv – Malvidin
Mv3glc – Malvidin 3-glucoside
na – Not attributed
nd – Not detected
ppm – Parts per million

NMR – Nuclear Magnetic Resonance

ROS – Reactive oxygen species

Pg – Pelargonidin

Pn – Peonidin

Pt – Petunidin

s – Singlet

Scp - Syringylcatechinpyrylium

rt – Retention time

Trolox - 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid

UV – Ultraviolet

Vis – Visible

δ – Chemical shift

λ_{max} – maximum absorption wavelenght

Scientific Communications and Thesis Organization

This dissertation integrates results that were published in international scientific conferences through panel and oral communications and in international journals with known scientific recognition:

Papers published on international scientific journals

1. **Sousa, A.**, Fernandes, A., Mateus, N., de Freitas, V. (2012) Synthesis and Structural Characterization of Oaklin-Catechins. *J. Agric. Food Chem.* 60(6): 1528–1534. doi:10.1021/jf204408p
2. **Sousa, A.**, Mateus, N., de Freitas, V. (2012) A novel reaction mechanism for the formation of deoxyanthocyanidins. *Tetrahedron Lett.* 53(10): 1300-1303. doi:10.1016/j.tetlet.2012.01.006
3. **Sousa, A.**, Petrov, V., Araújo, P., Mateus, N., Pina, F., de Freitas, V. (2013) Thermodynamics, Kinetics and Photochromism of Oaklins- a Recent Family of Deoxyanthocyanidins. *J. Phys. Chem. B.* 117(6): 1901-10. doi:10.1021/jp3110216.
4. **Sousa, A.**, Araújo, P., Mateus, N., de Freitas, V. (2013) Deoxyvitisins- a new set of pyrano-3-deoxyanthocyanidins. *Tetrahedron Lett.* 54(35): 4785-4788. doi:10.1016/j.tetlet.2013.06.135
5. **Sousa, A.**, Cabrita, L., Araújo, P., Mateus, N., Pina, F., de Freitas, V. (2014) Color stability and spectroscopic properties of deoxyvitisins in aqueous solution. *New J. Chem.*, 38(2): 539-544. doi:10.1039/c3nj01271a
6. **Sousa, A.**, Araújo, P., Cruz, L., Brás, N. F., Mateus, N., de Freitas, V. (2014) Evidence for copigmentation interactions between deoxyanthocyanidin derivatives (oaklins) and common copigments in wine model solutions. *J. Agric. Food Chem.* 62(29): 6995–7001. doi:10.1021/jf404640m
7. **Sousa A.**, Araújo, P., Azevedo, J., Cruz, L., Fernandes, I., Mateus, N., de Freitas, V. (2015) Antioxidant properties of 3-deoxyanthocyanidins. Submitted to *Food Chemistry*.

Oral communications

1. **Sousa, A.**, Fernandes, A., Mateus, N., de Freitas, V. "Synthesis and structural characterization of oaklins dimers".
   "9th Symposium International d'Oenology", Bordeaux, France, 15-17 June 2011.
2. **Sousa, A.**, Petrov, V., Araújo, P., Mateus, N., Pina, F., de Freitas, V. "Thermodynamics, Kinetics, and Photochromism of Oaklins- A Recent Family of Deoxyanthocyanidins".
   "8th In Vino Analytica Scientia", Reims, France, 2-5 July 2013.

Panel communications

1. **Sousa, A.**, Fernandes, A., Mateus, N., de Freitas, V. "Synthesis and structural characterization of oaklins dimers"
   "9th Symposium International d'Oenology", Bordeaux, France, 15-17 June 2011
   "34th World Congress of Vine and Wine", Porto, Portugal, 20-27 June 2011
   "XXII Encontro Nacional da SPQ", Braga, Portugal, 3-6 July 2011
   "In Vino Analytica Scientia", Graz, Austria, 20-23 July 2011
2. **Sousa, A.**, Mateus, N., de Freitas, V. "A novel reaction mechanism for the formation of deoxyanthocyanidins"
   "XXVI International Conference on Polyphenols", Florence, Italy, 22-26 July 2012
3. **Sousa, A.**, Araújo, P., Mateus, N., de Freitas, V. "Deoxyvitisins: a new set of pyrano-3-deoxyanthocyanidins"
   "10º Encontro Nacional de Química Orgânica", Lisbon, Portugal, 4-6 September 2013
   "7th International Workshop on Anthocyanins", Porto, Portugal, 9-11 September 2013
4. **Sousa, A.**, Petrov, V., Araújo, P., Mateus, N., Pina, F., de Freitas, V. "Thermodynamics, Kinetics, and Photochromism of Oaklins- A Recent Family of Deoxyanthocyanidins".
   "XXIII Encontro Nacional da SPQ", Aveiro, Portugal, 12-14 June 2013.

General Introduction

The evolution process of the Plant Kingdom has led several plants to adopt defense mechanisms against agressions of their environment, leading to changes in their ability to synthethize and use chemical compounds that control their growth and development. Some of these compounds act as toxins against pathogenic agents or herbivores and others attract symbionts and other life beings required for procreation. The need for these chemical responses to changing environments has originated over time an enormous structural diversity on the largest groups of secondary metabolites that include alkaloids, terpenoids and other polyphenolic compounds. The latter assume a great importance, playing a range of diverse roles, such as the defense against agressors, flowers and fruits coloring, attraction of symbionts and the regulation of the cellular growth and maturation.

Polyphenols belong to a class of chemical compounds, which feature a benzene system with one or more hydroxyl groups, which in their turn may be methylated or glucosylated. They can be divided into several groups according to their properties, but are generically classified as non-flavonoids, including phenolic acids and other derivatives such as stilbenes, or flavonoids, which include flavonols, flavones, anthocyanins and others.

Polyphenols assume an important role in food matrixes and namely in wines, contributing directly to their organoleptic properties such as color and flavor. Anthocyanins, for example, are responsible for the majority of red/violet color in vegetables, while tanins are responsible for the adstringency and bitterness in food. In wine, throughout the fermentation and aging processes, these compounds undergo major changes, quantitatively and structurally, usually leading to the formation of new products with different physical-chemical properties from their precursors.

Some of these compounds, such as anthocyanins and derivatives present intense colors and are relatively stable in aqueous solutions. This feature has given them the potential advantage to act as viable commercial food colorants, which has consequently led to advances in the research developed in their chemistry over the years, in particular, the search for new colorants with significant stability.

Furthermore, these compounds present interesting photochemical behaviors in certain conditions and may undergo reversible and non-reversible structural transformations, according to pH, concentration, solvents, light stimulus, etc. These recent discoveries have widen up the range of their applications which now extend to their use as hair dyes, laser dyes, sensitizers for solar cells, and molecular-level memory systems.

Besides their obvious use and potential in the industry of food colorants, cosmetics, energy transformation and information systems, they also have been studied for their antioxidant properties and regarded as promising health promoting agents and of great nutritional value in diets.

1. Flavonoid and non-flavonoid compounds

Phenolic compounds present as its base structure an aromatic ring, which can be attached to one or more hydroxyl groups, leading to a wide range of compounds. Polyphenols can be divided into two large groups: flavonoids, which are part of flavanols, flavonols, flavanononones, flavones and anthocyanins (Fig. 1); and non-flavonoids, which include phenolic acids (benzoic and cinnamic), and other phenolic derivatives such as stilbenes (e.g. resveratrol).

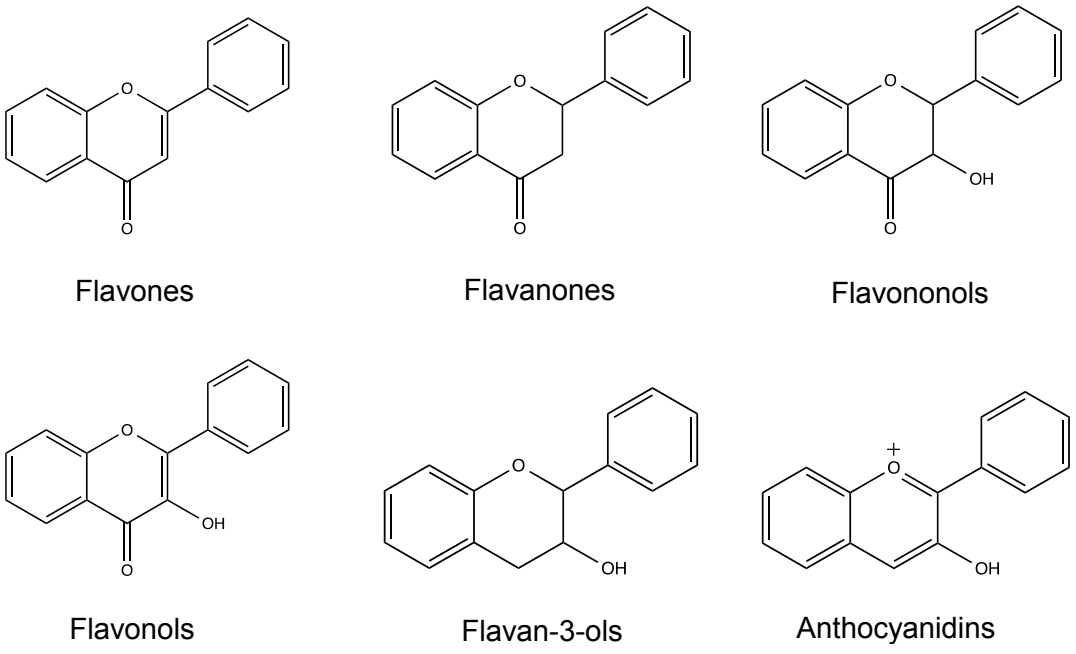


Fig. 1 - Subclasses of flavonoids.

Flavonoids are compounds widely distributed in the plant kingdom, are present in fruits, leaves, seeds and other plant parts in the form of glycosides and aglycones. They are compounds with a relatively low molecular weight, consisting of 15 carbon atoms arranged in a C6-C3-C6 configuration. The chemical structure of flavonoids consists of two aromatic rings, designated ring A and B, joined by three carbons which form an heterocyclic ring, called ring C (Merken and Beecher 2000) (Fig. 2). Substitutions on ring C result in important classes of flavonoids such as flavonols, flavones, flavanones, flavanols (e.g. catechins), isoflavones and anthocyanidins (Fig. 3). Substitutions in rings A and B give different compounds within each class of flavonoids. These substitutions may include oxygenation, alkylation, glycosylation, acylation and sulfation (Hollman and Katan 1999).

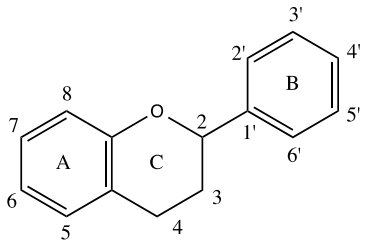
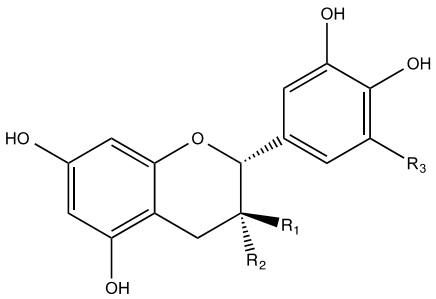


Fig. 2 - Base structure of flavonoid compounds (2-phenylbenzopyran, commonly named flavanic nucleous).

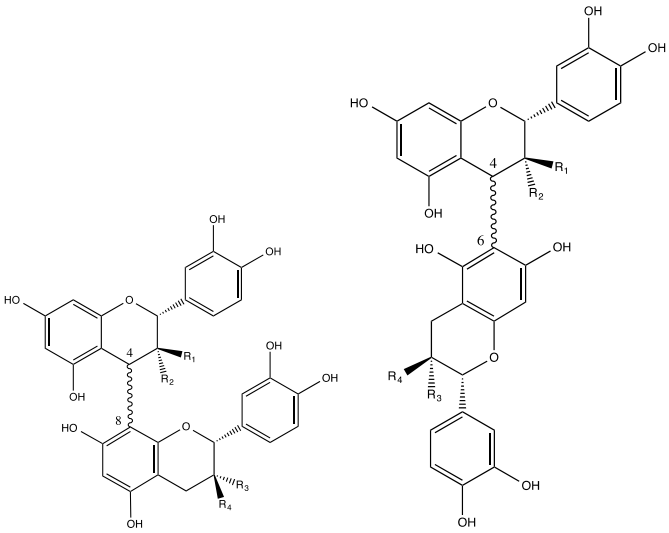
Taking a closer look at the group of flavanols, their types of structures in nature differ in the stereochemistry of the asymmetric carbons of the piranic ring (C2 and C3) as well as in the degree of hydroxylation of the rings A and B (Fig. 3). The monomeric flavanols most common in plants, and in particular the species *Vitis vinifera*, are hydroxylated at positions 5 and 7 of the ring A, differing only in the number of hydroxyl groups on ring B and on the stereochemistry of carbon 3 from ring C.



Flavanol	**R1**	**R2**	**R3**
(+)-catechin	OH	H	H
(-)-epicatechin	H	OH	H
(+)-gallocatechin	OH	H	OH
(-)-epigallocatechin	H	OH	OH

Fig. 3 - 3-flavanol monomers (catechins and gallocatechins).

In nature, flavanols also exist in polymerized forms, called proanthocyanidins (or condensed tannins). This family of polymers differ in the nature of the constituent monomer units (procyanidins consist of catechins, while prodelphinidins consist of gallocatechins), in the type of interflavanolic bond between these units (e.g. C4-C6 or C4-C8 bonds which are characteristic of dimeric procyanidins of type B, Fig. 4) and in their degree of polymerization (dimers, trimers, oligomers and polymers) (Batesmith 1954, Haslam 1996).



Flavanol	R1	R2	R3	R4
B1	OH	H	H	OH
B2	OH	H	OH	H
B3	H	OH	H	OH
B4	H	OH	OH	H
B5	OH	H	OH	H
B6	H	OH	H	OH
B7	OH	H	H	OH
B8	H	OH	OH	H

Fig. 4 - Type B dimeric procyanidins.

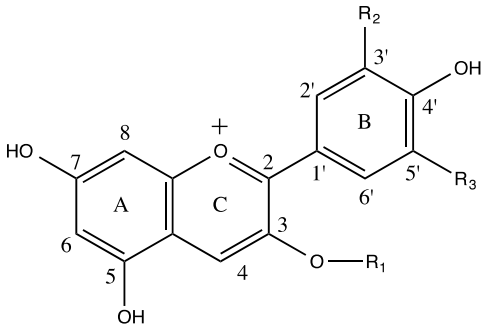
2. Anthocyanins

Anthocyanins (from the Greek *anthos* meaning flower and *kyanos* which means blue) are the most abundant and important pigments of plants, fruits and vegetables, being responsible for the extensive range of colors presented by these products.

They are water-soluble pigments and are present in the vacuole of the epidermal cells of plants, generally giving them a red, violet or blue color, according to the vacuolar pH. Anthocyanins can be found in all plant tissues, leaves, roots, stems, flowers and fruits. They are not found in animals, plants or marine organisms. Their role as coloring agents constitutes an important factor in animal attraction, aiding in pollination, and having a considerable value in this co-evolution of plant-animal interactions. Besides the coloring functions in plants, they are particularly important in UV protection, defense against pathogens and insect attacks (Stintzing and Carle 2004).

Its structure corresponds to the glucosylated form of anthocyanidins, which comprise the flavylium cation polyhydroxylated and/or methoxylated. The flavylium cation corresponds

to a benzene system formed by the rings A, B and C and anthocyanidins differ in the degree of hydroxylation and/or methoxylation of the B ring (Fig. 5).



Anthocyanidin	R1	R2	R3	λ_{max} (nm)
Pg	H	H	H	520
Cy	H	OH	H	535
Df	H	OH	OH	546
Pn	H	OCH_3	H	532
Pt	H	OCH_3	OH	543
Mv	H	OCH_3	OCH_3	542
Anthocyanin				
Pg 3-glc	Glc	H	H	516
Cy 3-glc	Glc	OH	H	530
Df 3-glc	Glc	OH	OH	543
Pn 3-glc	Glc	OCH_3	H	536
Pt 3-glc	Glc	OCH_3	OH	546
Mv 3-glc	Glc	OCH_3	OCH_3	546

Fig. 5 - Anthocyanins (flavylium cation) and their λ_{max} values in acidic methanol (adapted from (Stintzing and Carle 2004)).

In fruits, anthocyanins are found in the glycosylated form, though in other contexts they may exist in the non-glycosylated form, which is known as anthocyanidins (aglycones). Several anthocyanidins have been identified in nature, but only six are present in superior plants: cyanidin, delphinidin, malvidin, pelargonidin, peonidin and petunidin (Francis 1989). Its different pattern of hydroxylation and methoxylation, leads to the appearance of a large variety of colors ranging from orange-red, for example in pelargonidin, to the blue-violet in delphinidin at acidic pH. Generally, the hydroxylation of ring B induces bathochromic deviations (higher wavelength values of the maximum absorbance), while the methylation of these hydroxyl groups reversed this trend (Brouillard 1983, Stintzing and Carle 2004). The glucosylation of anthocyanidins (anthocyanins) leads to a greater stability and solubility of these compounds in aqueous solutions also causing slight changes in their maximum absorbance wavelengths. Fruit anthocyanins are generally O-glucosylated in the carbon of position 3, but may also be glucosylated at positions 5, 7, 3' or 5'. The most common sugars are glucose, ramanose, galactose, xylose and arabinose. The glucosidic fragments can also be esterified with aliphatic acids such as acetic acid, or aromatic acids, such as coumaric and caffeic acid (Francis 1989) (Fig. 6).

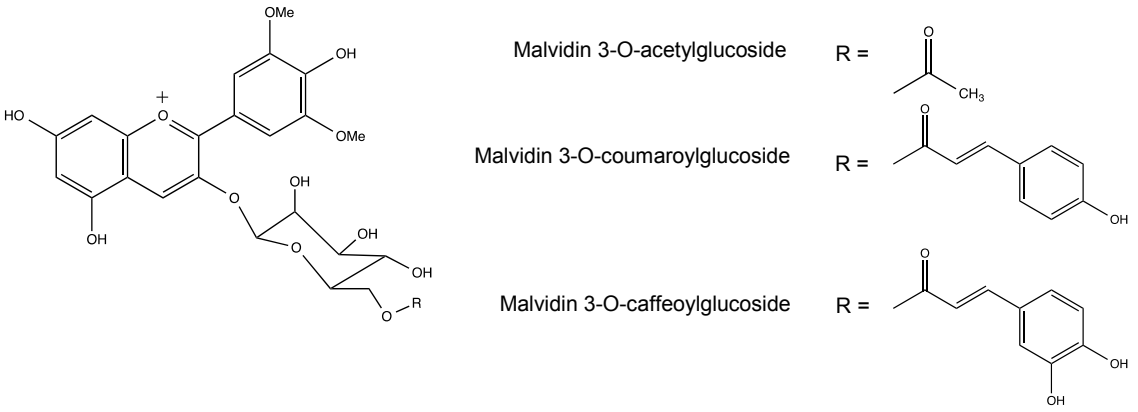


Fig. 6 - Acylated esters of malvidin 3-glucoside.

All this structural diversity contributes to the high number of anthocyanins that are known today, and it is estimated that there are over four hundred anthocyanins in nature (Francis 1989). However, despite this multitude of compounds, they are still insufficient to justify all the variety of colors displayed by flowers and fruits, and it is necessary to take into account other matters that might explain the different chromaticity such as the pH, self-association phenomena and copigmentation.

In addition to the diversity of natural anthocyanins, several other pigments are found in processed foods, such as wine, olive oil, chocolate, tea, etc. Some of the compounds that have been detected in wine are anthocyanin pyruvic derivatives (Fig. 7) (Fulcrand, Benabdeljalil 1998, Mateus, Silva 2003). These compounds exhibit an orange color, are more stable and present a higher color intensity compared to their anthocyanin precursors (Oliveira, Mateus 2009, Carvalho, Oliveira 2010).

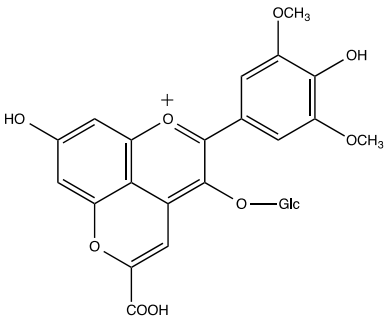


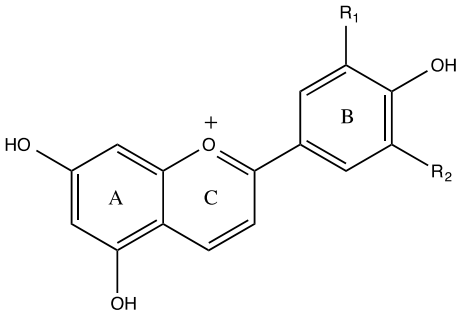
Fig. 7 - Malvidin 3-glucoside pyruvic derivative.

The diversity of colors exhibited by natural pigments and their derivatives is the reason why they have been studied so extensively, since there is a vast range of technological applications, particularly in the food industry. However, a major obstacle in the application of anthocyanins in food matrices results from their instability to pH and temperature fluctuations.

3. Deoxyanthocyanidins

Deoxyanthocyanidins are yellowish pigments found in several food plants such as corn, black tea leaves, and sorghum. Sorghum is one of the most important cereal crops in the world and is rich in 3-deoxyanthocyanidins, particularly luteolinidin and apigeninidin (Sweeny and Iacobucci 1977, 1981). Tricetinidin is another natural deoxyanthocyanidin and is found exclusively in black tea during the fermentation stage by the enzymatic oxidation of epigallocatechin.

Deoxyanthocyanidins are considered the chemical ancestors of anthocyanins, the ubiquitous water-soluble pigments that are found in flowers and fruits and are responsible for their impressive colors. Opposite to anthocyanins, these pigments lack the glycosylated group (Fig. 8).



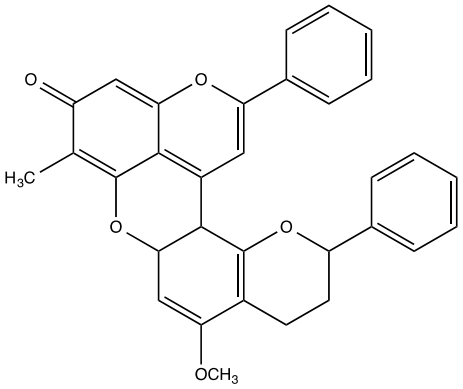
Deoxyanthocyanidin	R1	R2
Apigeninidin	H	H
Luteolinidin	OH	H
Tricetinidin	OH	OH

Fig. 8 – Deoxyanthocyanidins' structure.

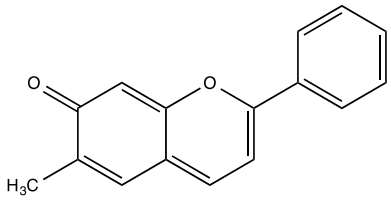
Another natural source of deoxyanthocyanidins is a peculiar type of trees' resin. It's called Dragon's blood and is a natural resin, with a deep, rich red colour, that is obtained from several trees, namely from *Dracaena draco* and *Dracaena cinnabari*, which belong to the *Liliacae* family. The resin appears in injured areas of the plant, and it has been used for centuries for diverse medical and artistic purposes (Melo, Sousa 2007). Some of the molecules responsible for the red colour of the resin obtained from the palm tree *Daemonorops draco* are represented in Fig. 9 (Brockmann and Haase 1936, Brockmann and Junge 1943).

Open chalcone forms of the common anthocyanins are assumed to be crucial in reactions leading to irreversible degradation of anthocyanins, particularly under weakly acidic to weakly alkaline solution conditions (Mazza and Brouillard 1987, Francis 1989, Cabrita, Fossen 2000, Torskangerpoll and Andersen 2005, Sadilova, Carle 2007). However, the natural yellow deoxyanthocyanidins are much more stable in slightly acidic solutions than anthocyanins and anthocyanidins, which points to the potential advantage of this type of compounds as viable commercial food colorants, and justifies the research developed in the chemistry of 3-deoxyanthocyanins and, in particular, the search for new colorants with significant stability (Iacobucci and Sweeny 1983, Dangles and Elhajji 1994, Khalil, Baltenweck-Guyot 2010). Furthermore, more studies have demonstrated other potential applications for these compounds, such as their use as hair dyes, laser

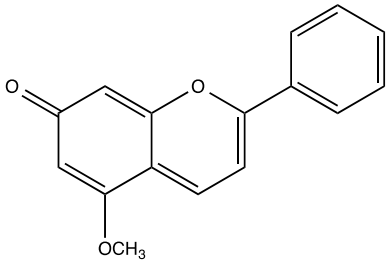
dyes, sensitizers for solar cells, and molecular-level memory systems (Czerney, Graness 1995, Cherepy, Smestad 1997, Roque, Lodeiro 2002).



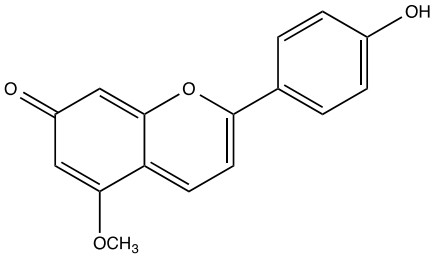
Dracorubin



Dracorhodin



Nordracorhodin



Dracoflavylium

Fig. 9 - Quinoidal bases reported for the dyes responsible for the red colour in Dragon's blood resin.

Despite the interest in these types of compounds, few synthetic approaches have been made toward deoxyanthocyanidins and the procedures described in the literature were complex (Pratt and Robinson 1922, 1923, Pratt, Robinson 1924, Pratt and Robinson 1925, Sweeny and Iacobucci 1977, Kuhnert, Clifford 2001). Only in recent years attempts have been made to synthesize these compounds with simpler methods (Mas 2003, Chassaing, Kueny-Stotz 2007, Kueny-Stotz, Isorez 2007).

4. Physico-chemical properties of anthocyanins and deoxyanthocyanidins

4.1 Flavylium reaction network

In order to gain a deeper understanding of the flavylium chemistry, it is convenient to summarize the current state of the art for flavylium compounds.

Fig. 10 summarizes the network of chemical reactions involving flavylium compounds in acidic to neutral pH values. The flavylium cation, AH^+, is the predominant species in the equilibrium under sufficiently acidic conditions. When the pH is raised the flavylium cation is involved in two parallel reactions: (i) deprotonation to form the quinoidal base A; (ii) hydration in position 2 followed by proton loss to give the hemiketal B. The proton transfer is faster than the hydration but in many cases the other species (B, Cc, Ct) at the equilibrium are more stable than A. This means that A can be formed as a kinetic product, and later totally or partially disappears to yield the thermodynamic equilibrium. The *cis*-chalcone (Cc) is formed from B by a tautomeric process and the *trans*-chalcone (Ct) forms via the isomerisation of the former. The colored species are AH^+ and A that may appear as red and purple/blue in common anthocyanins, respectively. Photochemistry arises from the *trans* to *cis* isomerisation, and depending on pH leads to AH^+ and A. At equilibrium, the system behaves as a single acid–base equilibrium between the flavylium cation and a conjugate base, CB, defined as the sum of the concentrations of the other species in the network,

$$[CB] = [A] + [B] + [Cc] + [Ct]$$

$$AH^+ + H_2O \rightleftharpoons CB + H_3O^+ \qquad K'_a = K_a + K_h + K_h K_t + K_h K_t K_i$$

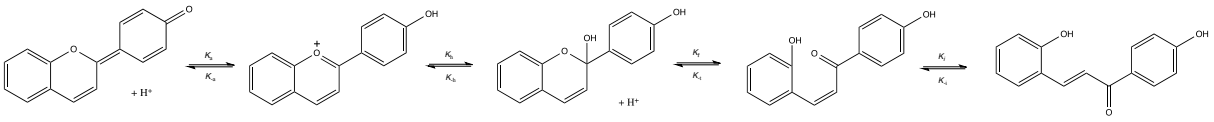


Fig. 10 – Flavylium network of chemical reactions.

It is now known that almost all flavylium compounds, independently of being natural or synthetic, follow the framework shown in Fig. 10. However, depending on the

substituents, the pH dependent mole fraction distribution in the equilibrium (thermodynamic) and the rates of conversion between the different species (kinetics) in the network are different. For example, in the most common anthocyanins the major product of the CB is the hemiketal, and the *cis* and *trans* chalcones are minor products. The same usually occurs in 3-substituted flavylium compounds, while for those lacking the 3 and 5 substituents (such as deoxyanthocyanidins), *trans*-chalcone is the counter part of the flavylium cation. On the other hand, 4-substituted flavylium compounds (e.g. pyranoanthocyanins) generally equilibrate between the flavylium cation and the quinoidal base. The base is also the major species at the equilibrium in 5-substituted flavyliums. Depending on pH it is also possible to observe the formation of *trans*-chalcones in anthocyanidins but the hydroxyl group in position 3 makes these unstable, decomposing into irreversible products (Furtado, Figueiredo 1993). In other words, distinct behaviors are observed according to the family of flavylium substituents. This was an additional drawback to early researchers to establish a common pattern as we have nowadays.

4.2 Deoxyanthocyanidins

The effect of substitution on the stability of 3-deoxyanthocyanidins was studied by Sweeny and Iacobucci. They studied several 3-deoxyanthocyanins and observed an unusually high stability at neutral and basic pH values.

In other works, the acidity constants of apigeninidin (Brouillard, Iacobucci 1982) and luteolinidin (Melo et al. 2007) were determined. In both cases, the mole fraction distribution of the base at pH = 5–6 was very high.

Another interesting observation regarding many of the contemporary works dealing with the compounds that give colour to dragon's blood resin is that they are the quinoidal bases of a flavylium cation (Fig. 9). Similar to other hydroxyflavylium compounds, the absorption by the base is red shifted in comparison with the flavylium cation and as a consequence, the quinoidal bases responsible for the red colour in dragon's blood become yellow in acidic medium due to the colour of the flavylium cation.

4.3 Self-association of anthocyanins

One of the mechanisms for color preservation consists on the formation of anthocyanins' aggregates, which become noncovalently grouped and vertically stacked (Fig. 11). This

self-association is explained by hydrophobic interactions between the aromatic rings, forming a complex with a chiral packing geometry, in which the axes of the superimposed structures form angles ranging between 0° and 90º (Hoshino 1991).

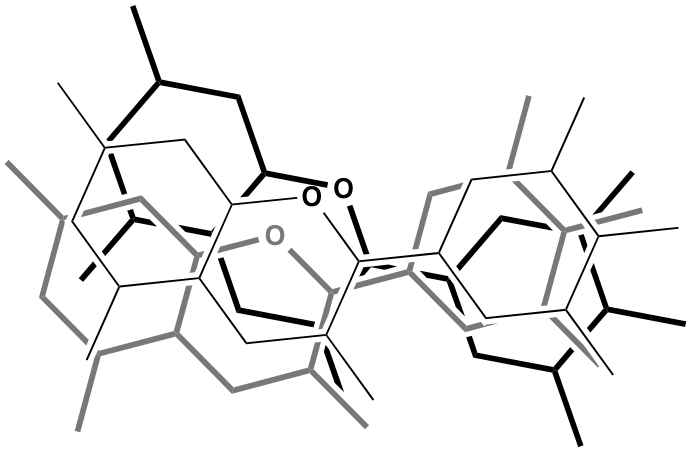


Fig. 11 - Chiral packing model on anthocyanins' self-association phenomenon (adapted from (Hoshino 1991)).

In this model there is a formation of an extensive hydrophobic core surrounded by glycosidic residues that make this entire complex water-soluble, while protecting the ring of the flavylium cation from a water nucleophilic attack, thus enabling stabilization against color loss. Given that this type of interaction is very strong, in the usual concentrations of these pigments in petals and flowers, this self-association phenomenon of anthocyanins contributes significantly to the colors displayed *in vivo* (Hoshino 1991).

4.4 Intramolecular copigmentation

Another phenomenon, which contributes to the stabilization of the color, is the intramolecular copigmentation that helps to stabilize the color of anthocyanins for slightly acidic to neutral pH values. Some anthocyanins are acylated with hydroxycinnamic acids and their phenolic groups form a sort of "sandwich" with the aromatic core of the anthocyanin (Fig. 12). This phenomenon consists of π-π hydrophobic intramolecular interactions between the two phenolic groups rich in electrons and the electron deficient core of the anthocyanin (the flavylium cation or the quinoidal base). The anthocyanin is

thus protected from the attack of water at higher pH values, contributing to a stabilization of the color (Kondo, Yoshida 1991).

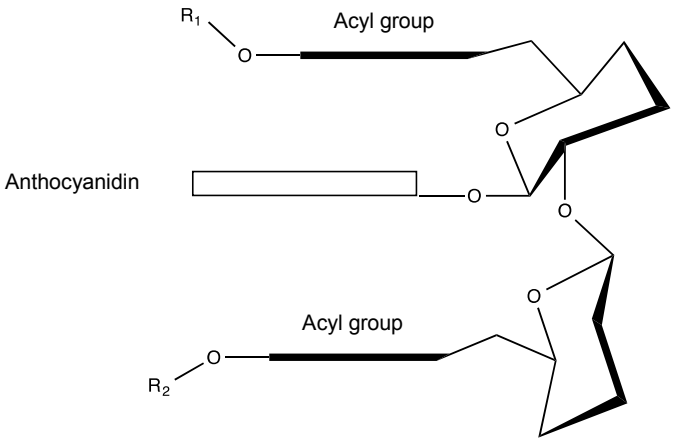


Fig. 12 - Representative scheme of the intramolecular copigmentation (adapted from (Kondo et al. 1991)).

4.5 Intermolecular copigmentation

Anthocyanins also present other mechanisms for the stabilization of its color in the presence of copigments, which are compounds that when isolated may even have very weak staining or may be even colorless, but when added to a solution of anthocyanins may contribute significantly to color retention. Analogously to what happens in intramolecular copigmentation, this phenomenon consists of hydrophobic interactions that take place in an aqueous solution between an electron-rich system (copigments) and an electron-deficient system (anthocyanin). These interactions thus protect anthocyanins from hydration and consequent color loss. This phenomenon is however dependent on various factors such as the type of copigments, the anthocyanin involved, pH, temperature, and even the presence of metals. Examples of copigments are flavonols, such as quercetin and hydroxycinnamic esters, such as glucose (Haslam 1998) (Fig. 13).

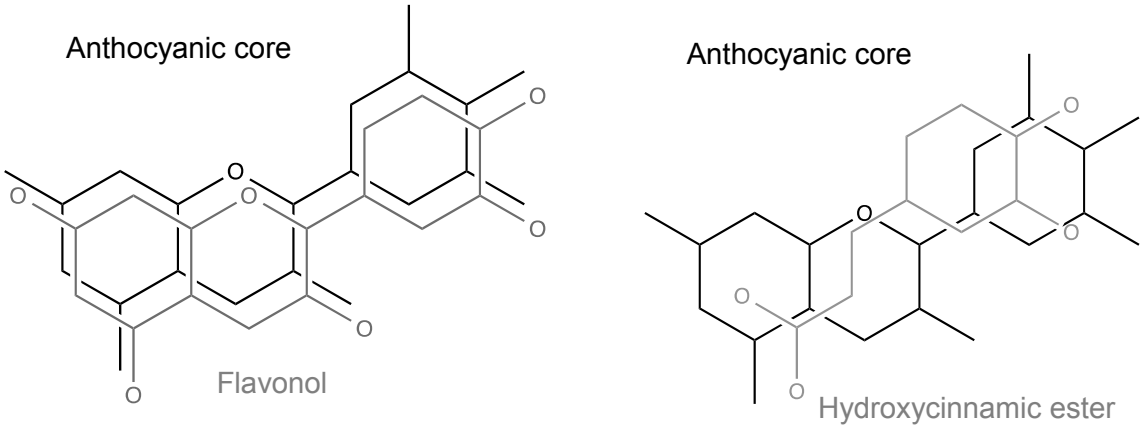


Fig. 13 - Examples of intermolecular copigmentation (adapted from Haslam, 1998).

5. Importance of the reactions between polyphenolic compounds

Polyphenolic compounds may react with each other and with other compounds, leading to structural changes and the subsequent modification of organoleptic properties in food. Flavanols, for example, have two hydroxyl groups in carbons C5 and C7 of ring A that induce formal negative charges by mesomeric donor effect in carbons C6 and C8, which conferrs a certain nucleophilic character to this ring. This characteristic is responsible for many already described reactions that occur involving these compounds.

In wine, for example, reactions occurring between anthocyanins and flavanols mediated by aldehydes, result in the formation of anthocyanin-flavanol adducts with alkyl/aryl/furanyl bridges derived from the aldehydes (Fig. 14). These aldehydes may stem from the fermentation or from the transfer of compounds from the wood during the wine aging process (Timberlake and Bridle 1976, Rivas-Gonzalo, Bravo-Haro 1995, Pissarra, Lourenco 2004, 2005).

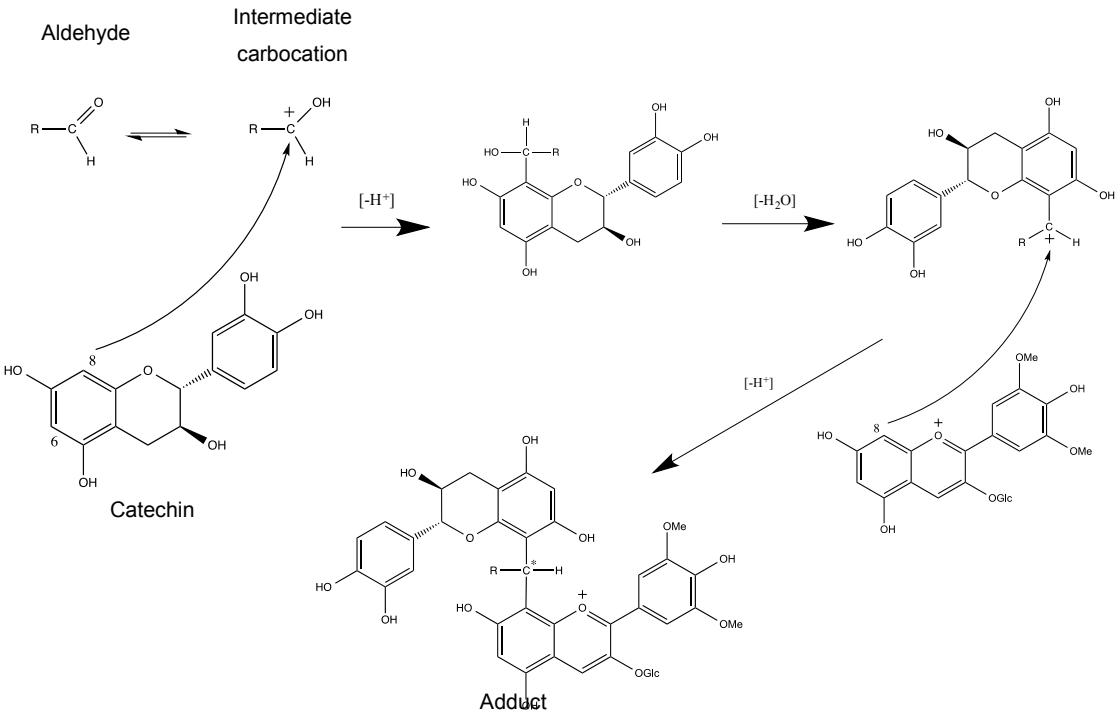


Fig. 14 - Formation mechaninsm of malvidin 3-glucoside-alkyl/aryl-catechin adducts (adapted from (Bendz, Martenss 1967)).

Regarding the mechanism, the aldehyde in acidic solution gives the carbocation by protonation of the carbonyl group. This cation will induce an electrophilic attack to the catechin ring, preferably in the position of carbon 8, since the negative formal charge is more delocalized for this carbon compared to carbon 6 (Bendz et al. 1967). An intermediate compound is formed, which then reacts with malvidin 3-glucoside yielding new adducts. Diastereoisomers (R and S) are also formed due to the presence of an asymmetric carbon in the interflavanoid bond (Bendz et al. 1967, Sousa, Mateus 2007).

These new compounds have different structural, chromatic and stability characteristics from their precursors and can somehow play an important role in the color evolution of wine aging.

The structure of anthocyanins also contains an important feature: the positive charge in the oxygen of ring C. This positive charge can be delocalized between carbons at position 2 and 4, conferring an electrophilic character to the pyranic ring. This fact provides two reactions, which define the color stability of anthocyanins. The first is the hydration reaction in the C2 position with the formation of hemiketal, as mentioned before and explained in detail; the second is the addition of bisulfite in the C4 position (Cheminat and Brouillard 1986, Berke, Cheze 1998, Escribano-Bailon, Alvarez-Garcia 2001) (Fig. 15). Sulfite is widely used in wine production, acting as an antioxidant agent

and inhibitor of growth of unwanted microorganisms (Bridle & Timberlake, 1966). Both reactions result in the discoloration of the solution, thus representing a very important topic of study on the stability of anthocyanin compounds.

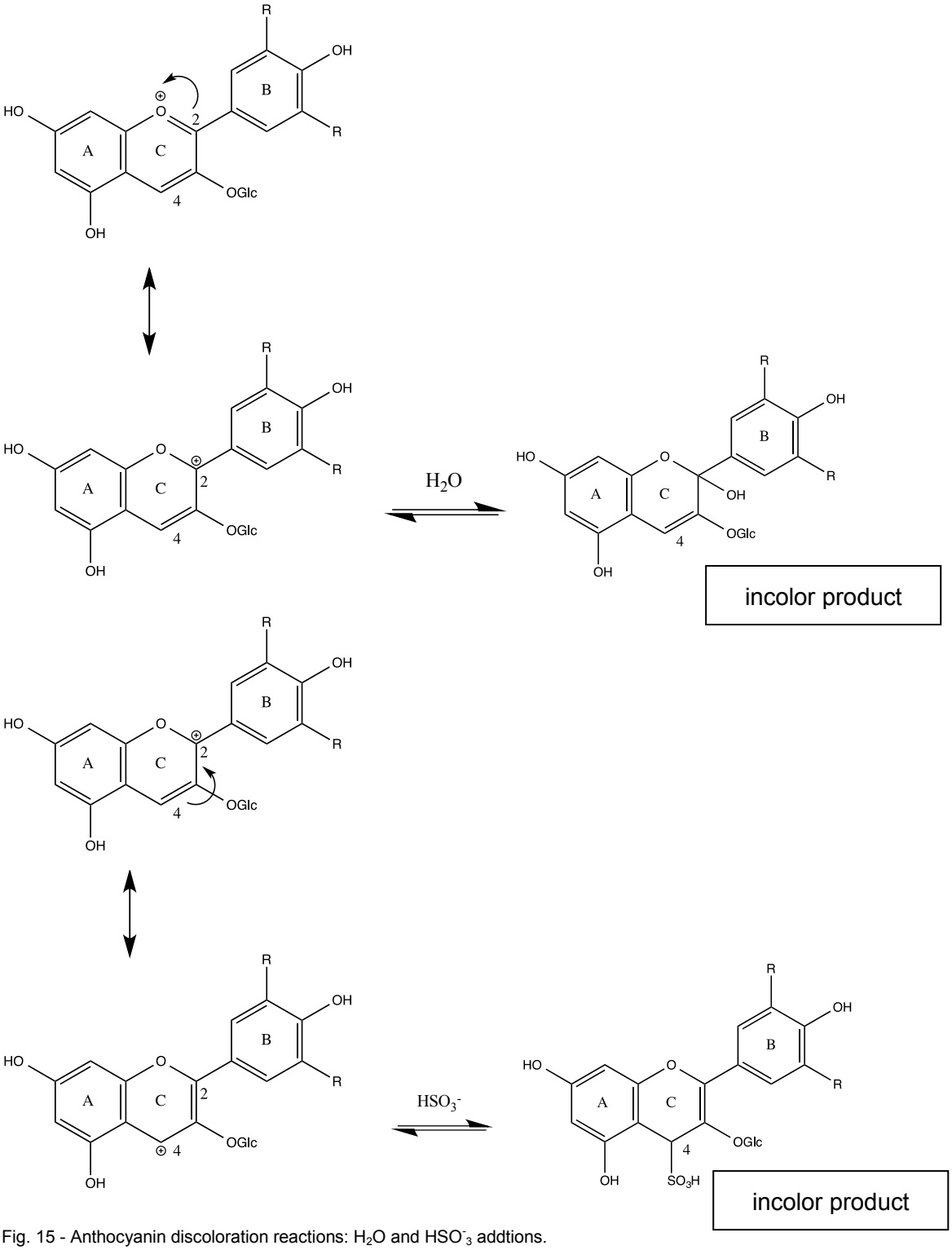


Fig. 15 - Anthocyanin discoloration reactions: H_2O and HSO^-_3 addtions.

In aqueous solution, SO_2 originates the bisulfite anion that rapidly and reversibly binds to carbon 4 of the flavylium cation, giving a colorless adduct. SO_2 can also bind to carbon 2 of the anthocyanin, also resulting in color loss.

The equilibrium constants of the reaction between bisulfite and anthocyanins are high, which is why small amounts of sulfite can bleach large quantities of anthocyanins (Timberlake and Bridle 1976). However, acidifying the medium, the equilibrium shifts toward the formation of the flavylium cation and the solution returns to the red color.

To summarize, it should be highlighted the importance of the reactions occurring between polyphenolic compounds, namely anthocyanin pigments, and its role in the evolution of food color. Particularly in wines, reactions that occur in the beginning of the winemaking process originate new pigments different from their precursors. And during the aging process other oxidation and polymerization reactions still contribute to the evolution of color of wines over the years. In this complex process, it should also be noted the importance of certain aldehydes and whose presence in wine is due to several factors, as by-products of alcoholic fermentation, resulting from the oxidation of higher alcohols, or by being extracted directly from the wood barrels where wines are stored (Wildenra.Hl and Singleto.Vl 1974, Canas, Belchior 2003, Pissarra, Mateus 2003). Wine storage also marks the importance of wood and the cooperage industry in the wine business; hence the composition of aldehydes can decisively influence the organoleptic properties of wines, especially color.

6. Oaklins

The use of oak wood (e.g., barrels and chips) in wine storage during the first years of aging is a procedure widely employed by winemakers, strengthening the wine organoleptic characteristics and contributing to its stability. Nevertheless, it must be carried out with caution to obtain better quality and well-balanced wines (Sanza, Dominguez 2004, Sanza, Domniguez 2004, Sanza, Escudero 2004).

Aging in oak wood allows wine to extract a series of phenolic and volatile compounds, depending upon the characteristics of the oak and the contact time between wine and wood. The factors that affect the pool of oak extractables are the species and

geographical origin of the wood (Chatonnet 1991, Miller, Howell 1992), as well as its processing, especially the method to obtain the staves, their seasoning, and the degree of oak toasting (Sefton, Francis 1993, del Alamo, Bernal 2000).

Among the potential compounds extractable from oaks, furanic (e.g., furfural and hydroxymethylfurfural), benzoic (e.g., hydroxybenzaldehyde and vanillin), and cinnamic aldehydes (e.g., coniferaldehyde and sinapaldehyde) are of particular interest because they can interact with some wine components (e.g., catechins and anthocyanins), hence contributing directly or indirectly to color and other sensory changes. As mentioned before, several studies have demonstrated that different aldehydes can serve as intermediaries in reactions involving catechin and anthocyanins or even react directly with catechin, yielding a variety of pigments with different color characteristics (Timberlake and Bridle 1976, Rivas-Gonzalo et al. 1995, Es-Safi, Cheynier 2000, Mateus et al. 2003). Recently, the formation of a new red/orange (λ_{max} at 500 nm) catechinpyrylium-derived pigment has been shown for the first time resulting from the direct reaction between catechin and sinapaldehyde, one of the main oak wood aldehydes (Fig. 16) (de Freitas, Sousa 2004).

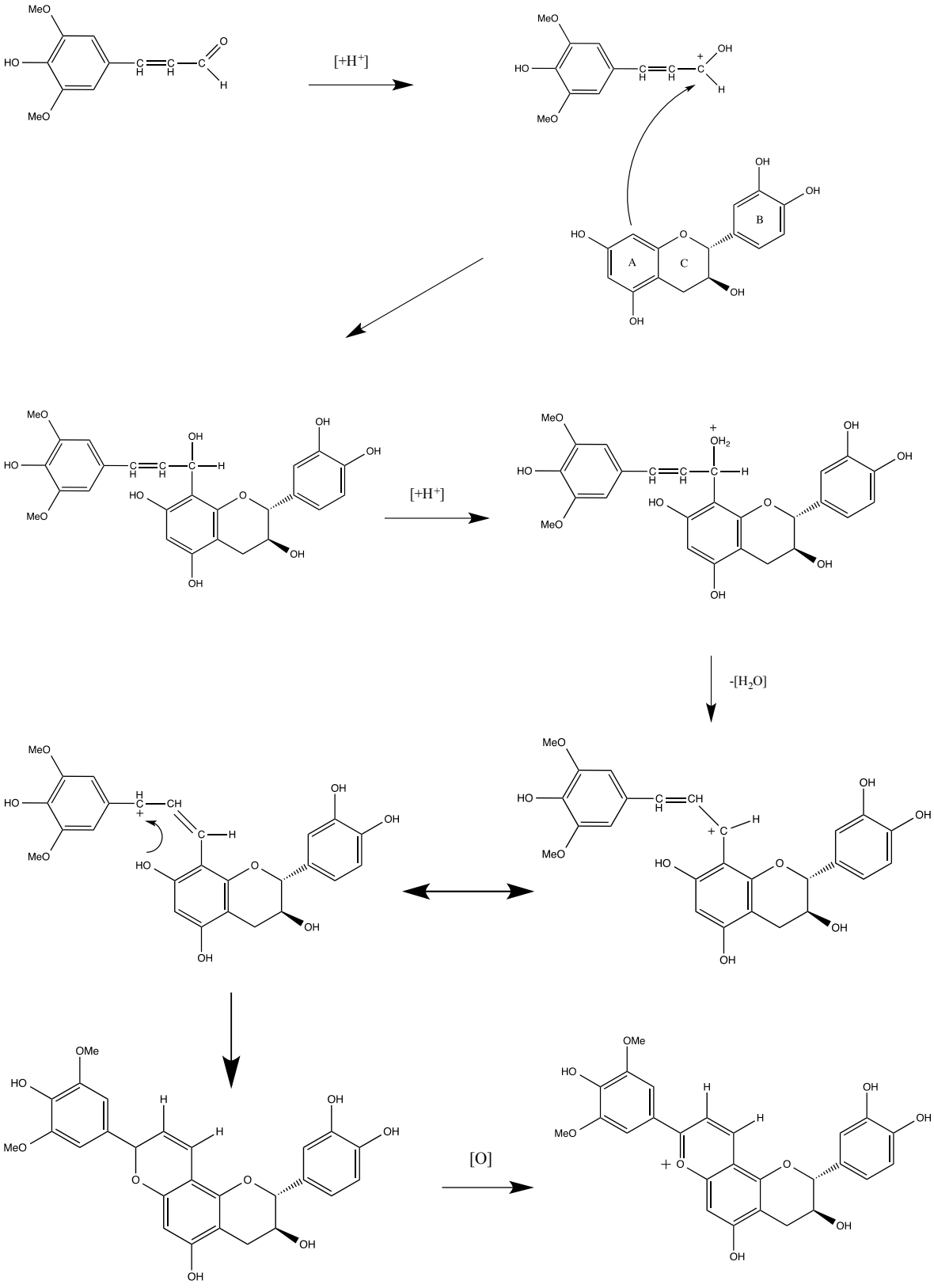


Fig. 16 - Hypothetic mechanism for the formation of catechin-pyrylium pigment obtained from catechin and sinapaldehyde (adapted from de Freitas, 2004).

In another work, it was reported the formation of brick-red pigments resulting from the reaction between catechin and an extract solution rich in oak aldehydes, especially sinapaldehyde and coniferaldehyde (Fig. 17).

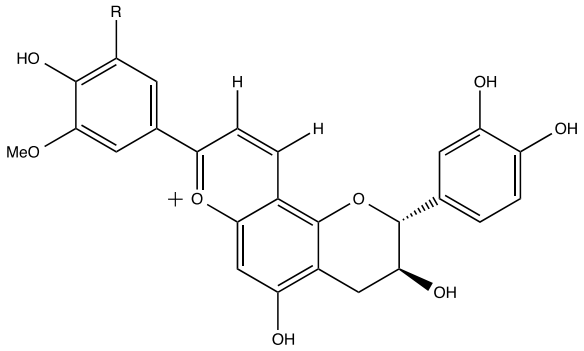


Fig. 17 - Structures of the catechinpyrylium pigments resulting from the reaction between catechin and coniferaldehyde (R = H, guaiacylcatechinpyrylium) or sinapaldehyde (R = OCH_3, syringylcatechinpyrylium) (adapted from Sousa, 2005).

The formation of a great variety of oaklins in the model solution with catechin and the oak extract was unambiguous, indicating that these pigments could also occur in wines aged in oak barrels.

The formation of syringylcatechinpyrylium and guaiacylcatechinpyrylium at pH 3.5 and 25°C was indicative of the possibility of the existence of this type of pigments in wines aged in oak barrels. To evaluate it, a 4-year-old commercial table red wine aged in oak barrels during a year was analyzed. Further fractionation of the wine was performed and the full mass chromatograms of these fractions displayed several molecular ion peaks supposedly corresponding to different oaklins, presenting molecular ions and retention times analogous to the ones corresponding to syringylcatechinpyrylium, guaiacylcatechinpyrylium, and guaiacylcatechinpyrylium/syringylcatechinpyrylium catechin adducts.

However and despite the extensive purification of the wine, the major part of these molecular ion peaks could not be individually selected because other compounds present in the obtained fractions were predominant. This did not allow the analysis of the fragmentation pattern of these pigments, thereby preventing further comparison and subsequent identification of these pigments with the various oaklins detected in model solution. This could be due to presumably low amounts of these pigments in wines, easily surpassed by other pigments such as the original anthocyanins and derived

compounds. Nonetheless, one of these pigments was individually and selectively detected in the wine.

These results demonstrated the existence of one of the main oaklins in a red wine. Therefore, it is expected that several oaklins can occur in different red wines aged in oak barrels. Because of their intense red/orange color and although probably not occurring in high amounts, their presence may somehow contribute to some color changes observed during wine aging in oak barrels.

This preliminary study confirmed the formation in model solutions of several red/orange pigments resulting from the reaction between catechin and aldehydes extracted from oak wood, namely, coniferaldehyde and sinapaldehyde. These pigments were all found to have a distinctive catechinpyrylium core and consequent related structural features, which lead to their designation as oaklins.

7. Antioxidant properties of anthocyanins and deoxyanthocyanidins

In addition to decisively influence the organoleptic properties of food, polyphenols also reveal important antioxidant properties.

Among various substances classified as antioxidants, there are for example vitamins, minerals, natural pigments and other vegatable compounds, and also enzymes, which block the effect of reactive oxygen species (ROS). These species negatively affect tissues, cells and genes contributing to the development of chronic diseases.

Antioxidants in food can normally act as inhibitors of radicals in chain reactions, as metal chelating agents, as inhibitors of oxidative enzymes and as cofactors of antioxidant enzymes (Huang 2005).

As a definition, an antioxidant is any substance that, when present at low concentrations compared to the oxidizable substrate, significantly delays or prevents oxidation of that substrate, and that after oxidation must be sufficiently stable so as not to trigger new

oxidation reactions (Shahidi, Janitha 1992). A compound may have an antioxidant action by inhibiting reactive oxygen species directly or by neutralizing free radicals. Antioxidants thus prevent the oxidation of other chemical substances, which occurs in the metabolic reactions or by exogenous factors such as ionizing radiations.

In the food industry, antioxidants also play an important role in food preservation by inhibiting lipid peroxidation and preventing food from becoming rancid, thus not loosing its nutritional and commercial value.

In short, regarding the nutritional aspect, polyphenols exert an important effect on the development of sensory characteristics such as color and flavor, while on the health perspective they contribute to the antioxidant defense of the body, preventing the occurrence of cardiovascular diseases (Williams, Spencer 2004).

7.1 Experimental methods to measure antioxidant properties

The chemical principles underlying the methods for the analysis of antioxidant properties depend on the reactions involved. Assays based on electron transfer reactions constitute a measure of the antioxidant capacity in the reduction of an oxidant, which changes color when reduced. This change is correlated to the antioxidant concentration and the color of the sample (Huang 2005).

7.1.1 DDPH – antiradical capacity

This method involves the use of a stable free radical, the DPPH (2,2-diphenyl-1-picrylhydrazyl), which reacts with the antioxidant in solution (Fig. 18). The reduction of DPPH concentration is monitored by the decrease in absorbance on a specific wavelength during the reaction. In its radical form, DPPH absorb at 515 nm, but upon reduction by an antioxidant or a radical species, the visible absorption at 515 nm disappears (Bondet, BrandWilliams 1997).

The ability to neutralize free radicals can thus be evaluated using DPPH as a free radical.

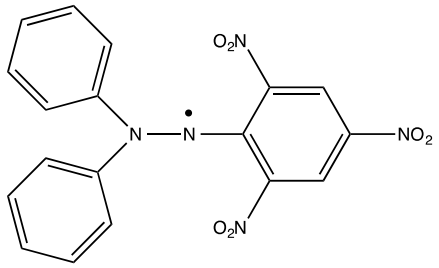


Fig. 18 - DPPH radical structure (2,2-diphenyl-1-picrylhydrazyl).

7.1.2 FRAP – reducing power

The FRAP (Ferric Reducing Ability Power) method consists in the reduction of ferric tripyridyltriazine complex $[Fe(III)\text{-}(TPTZ)_2]^{3+}$ to ferrous tripyridyltriazine complex $[Fe(II)\text{-}(TPTZ)_2]^{2+}$ by an antioxidant, normally in non-physiological conditions, at about pH 3.6 (Fig. 19) (Benzie and Strain 1996).

The formation of the reaction product, which has an absorption maximum at 593nm, can thus be measured by spectrophotometry. Its formation will reflect the reductive capacity of the antioxidant.

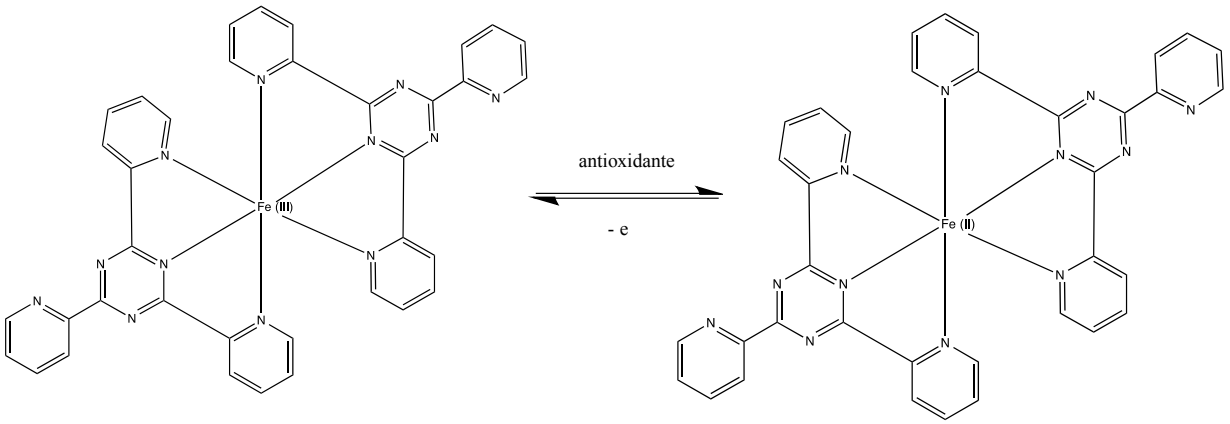


Fig. 19 - Redox reaction of ferric tripyridyltriazine to ferrous tripyridyltriazine.

7.1.3 Lipid peroxidation inhibition in liposomes

Most of the methods used to analyze anthocyanins' antioxidant properties focus on the different mechanisms of the antioxidant defense system, such as scavenging of oxygen and hydroxyl radicals. On the other hand, the use of liposomes allow the study of the

protection of a specific substrate by an antioxidant in a model biological membrane and has become a more promising method for the assessment of antioxidant properties relevant to human nutrition.

This method consists in using the azo compound AAPH (2,2'-Azobis(2-amidinopropane) dihydrochloride), which suffers thermal degradation and subsequently generates peroxyl radicals at a constant rate, thus inducing lipid peroxidation in liposomes. The reactions is conducted in the presence and absence of antioxidants and followed by measuring the rate of oxygen consumption with an oxygen electrode (Porto, Laranjinha 2003, Faria, Oliveira 2005).

8. Polyphenolic compounds in food

Many plant foods are rich in polyphenols. Foods and beverages known for their high content in polyphenols include fruits, vegetables, cereals and derived products such as beer, fruit juices, wine, tea and chocolate.

Depending on the diet, a person can consume from 100ng to several grams daily of polyphenolic compounds (Macheix 1990).

The association of a Mediterranean diet to a low incidence of cardiovascular diseases is well known and supported by several epidemiological studies (Hertog, Feskens 1993, Hollman and Katan 1999, Kaur and Kapoor 2001). This diet features a low consumption of foods with high values of saturated fat and calories, but rather presents a great consumption of foods high in complex carbohydrates, fiber, vitamins, minerals and numerous antioxidants, including polyphenols-rich foods.

The French Paradox is a classic example of this association: the population of a French region had a low incidence of cardiovascular diseases, despite their high consumption of saturated fats and smoking habits. This result was attributed to the regular and moderate consumption of red wine (Renaud and Delorgeril 1992).

Regarding the total daily intake of polyphenols it is estimated that one third consists of phenolic acids and two thirds of flavonoids (Scalbert and Williamson 2000). The

flavonoids most consumed are condensed tannins as well as anthocyanins, which justifies the interest in research of these compounds.

Concerning the beneficial effects of polyphenols on human health, one of the main questions resides in their bioavailability. This is because a compound may have great antioxidant capacity and other important biological activities *in vitro*, which will not be significant *in vivo* if little or no compound reaches the tissues / organs' target. Thus, after an estimated intake of foods rich in polyphenols, it is important to estimate and investigate their bioavailability.

Within the various groups of polyphenols, anthocyanins' bioavailability has been the most studied and currently it is known that the absorption of some of these compounds occurs in the small intestine (Del Rio, Borges 2010). It has been accepted that the absorption of anthocyanins occurs in the form of aglycones, after hydrolysis of glycosides.

Compounds that are not absorbed in the small intestine can be absorbed in the large intestine where the gut microflora can cleave the conjugated molecules. The resulting aglycones can still undergo ring opening leading to the formation of phenolic and hydroxycinnamic acids which can be absorbed and subject to phase II metabolism in the liver before being excreted.

Regarding the bioavailability of proanthocyanidins, existing literature is not as extensive. Some studies suggest that only oligomers of low molecular weight and degree of polymerization (monomers, dimers and trimers) are absorbed intact in the gastrointestinal tract (Deprez, Brezillon 2000, Holt, Lazarus 2002, Rasmussen, Frederiksen 2005) and the structures more complex would be degraded under the conditions of the stomach. However, other studies support that polymeric proanthocyanidins pass unchanged through the small intestine, being then degraded by the microflora phenolic acids in the large intestine (Deprez et al. 2000). The degradation products would include phenylacetic, phenylpropionic and phenylvaleric acids.

After the passage of the intestinal barrier by the proanthocyanidins or its derivatives formed during fermentation, these compounds arrive to the liver through the portal vein, where they are metabolized, probably hydroxylated, methylated or conjugated into sulfate esters or glucuronylated as with other flavonoids (e.g. anthocyanins).

Regarding the beneficial effects for the human health, several studies have been reported that prove this relationship. The antioxidant and anticancer abilities of

polyphenols have been proven countless times (Faria, Calhau 2006, Fernandes, Fernandes 2009, Faria, Pestana 2010). In addition, it has been observed a decrease of the risk of cardiovascular diseases (by the reduction of LDL oxidation and deacrease of serum cholesterol (Green and Jucha 1986), reduction of the risk of development of neurodegenerative diseases, particularly Alzheimer's and Parkinson's disease (Ehrnhoefer, Bieschke 2008, Wang, Ferruzzi 2012) and retardation of cellular aging (Howitz, Bitterman 2003).

Objectives

The purpose of this project was to investigate and study pigments with interesting color features and stability in aqueous solution that could have potential applications in food, health and other industries. Bearing this, several new pigments of the family of deoxyanthocyanidins were synthesized, isolated and structurally characterized. Their physico-chemical properties were thoroughly investigated as well as their stability in aqueous solution in the absence/presence of copigments. Finally, their antioxidant properties were also evaluated. During the project various issues and specific objectives were addressed:

Chapter 1

During wine aging in oak barrels several wood compounds (e.g. aldehydes) can interact with wine constituents like anthocyanins and catechins yielding new pigments that sometimes present interesting color features and are more stable than their precursors.

This work deals with the formation of new oaklin pigments resulting from a direct reaction of a procyanidin dimer B4 ((+)-catechin-(4−8)-(−)-epicatechin) with two cinnamic aldehydes, coniferaldehyde and sinapaldehyde in hydroalcoholic solutions. The focus of this work was to evaluate the importance that dimeric procyanidins and cinnamic aldehydes could have on the color evolution of wines during storage in oak barrels.

Chapter 2

Despite the interest in deoxyanthocyanidin-type of compounds, few synthetic approaches have been made toward their synthesis and the procedures described in the literature are rather complex.

The goal of this work was to test a simple synthesis method to form 3-deoxyanthocyanidins from the reaction between phloroglucinol with two cinnamic aldehydes, coniferaldehyde and sinapaldehyde, describing their chemical structure and mechanism of formation and evaluating the kinetics of formation of the reaction products under different conditions.

Chapter 3

Two oaklin compounds (guaiacylcatechinpyrylium and syringylcatechinpyrylium), which are formed in wine aged in oak barrels and a model deoxyanthocyanidin compound (deoxypeonidin) were synthesized.

Combining pH jump techniques with flash photolysis, the rate and equilibrium constants of the respective pH dependent network of chemical reactions were calculated and their photochromism was also investigated.

This work would allow a global understanding and comprehensive analysis of the stability of these types of deoxyanthocyanidins, comparing their behaviour and physico-chemical properties under different pH conditions and light stimulus with analogous compounds such as common anthocyanins and other flavyliums.

The ultimate purpose of this work was to evaluate and identify potential applications for these types of compounds, coming from the knowledge of their physico-chemical characteristics.

Chapter 4

Pyranoanthocyanins are anthocyanin derived pigments with an “extra” pyranic ring, which makes them much more stable towards pH variations and bleaching by SO_2 in comparison to the genuine anthocyanins. On the other hand, and opposite to anthocyanins, deoxyanthocyanidins also have an increased stability in slightly acidic solutions compared to anthocyanins.

Bearing this, the aim of this work was to synthesize compounds that would benefit from both structural features. These compounds would be called deoxyvitisins (or pyranodeoxyanthocyanidins) and would be obtained from the reaction between deoxyanthocyanidins and the reagents pyruvic acid, vinyloxy-trimethylsilane and acetone-1,3-dicarboxylic acid in separate model aqueous solutions.

Chapter 5

The aim of this work was to investigate the color stability and the network of chemical reactions occurring in aqueous solution upon pH variations for the deoxyvitisins synthesized in the previous work, comparing the results with a flavylium model

compound 4',7-dihydroxyflavylium and with values reported in the literature for the corresponding glycosidic analogs, vitisins.

Chapter 6

The importance and contribution of the copigmentation effect cannot be underestimated in the analysis of color changes in wine solutions.

The aim of this work was thus to study the copigmentation interactions possibly occurring between the oaklin compounds guaiacylcatechin-pyrylium (GCP) and syringylcatechin-pyrylium (SCP) with common copigments such as catechin, epicatechin, chlorogenic acid, epigallocatechin, and procyanidin B3, determining the respective copigmentation constants and evaluating the relationship between copigmentation ability and the structure of the complexes formed.

Chapter 7

The study of the antioxidant properties of six deoxyanthocyanidins (deoxypeonidin, deoxymalvidin, luteolinidin, apigeninidin, guaiacylcatechinpyrylium and syringylcatechinpyrylium) and an anthocyanin (cyanidin-3-glucoside) was carried out. The aim was to evaluate the relationship between the structure and the antioxidant properties of individual deoxyanthocyanidins, compared to a common derivative anthocyanin, cyanidin-3-glucoside. The ability of these compounds to inhibit lipid peroxidation in a liposome membrane system was examined by monitoring oxygen consumption and the antiradical and reducing capacities were determined using the DPPH and FRAP assay, respectively. Furthermore, the antiproliferative effects of deoxyanthocyanidins have been evaluated against two cancer cell lines from stomach (AGS, MKN-28) and one colon cancer cell (Caco-2), and compared with the effect of their anthocyanic forms.

Results

Chapter 1

Synthesis and Structural Characterization of Oaklin-Catechins

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In this work, the first author was responsible for undertaking all experimental activities with the assistance of MS and NMR technicians and guided by the scientific knowledge of the rest of the authors.

Synthesis and structural characterization of oaklin-catechins

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Condensation reactions of procyanidin dimer B4 with two representative oak wood cinnamic aldehydes (coniferaldehyde and sinapaldehyde) were conducted in wine-like model solutions. Coniferaldehyde led to the formation of guaiacylcatechin-pyrylium-catechin (GCP-catechin, 737 m/z), whereas sinapaldehyde led to the formation of syringylcatechin-pyrylium-catechin (SCP-catechin, 767 m/z). The former was also structurally characterized by 1D and 2D NMR, allowing an elucidation of the formation mechanism of these oaklin-catechin adducts and demonstrating the importance of procyanidins in the formation of colored compounds through the reaction with cinnamic aldehydes extracted from oaks during storage.

KEYWORDS: Red wine; aging; oak barrels; procyanidin B4; aldehydes; oaklin; NMR; mass spectrometry

- INTRODUCTION

Wine storage in oak barrels during the first years of aging is a common procedure in wine industry and the extraction of volatile and non-volatile compounds from the wood influences important characteristics in matured wine, namely aroma, taste and color (Jurd 1969, Chassaing, Lefeuvre 2010). The extraction of these compounds depends on the period of contact between wine and wood and on the chemical composition of the wood, which is affected by the species and origin of the trees, the seasoning of the staves, the age of the barrel and most important by the heat treatment or toasting of barrels (Chatonnet, Boidron 1989, Sefton et al. 1993, Vivas 1995). In this procedure, macromolecular components like lignins and polysaccharides of the wood are degraded into smaller compounds such as several aldehydes (Nonier, Vivas 2006) (eg. furfural, hydroxymethylfurfural, hydroxybenzaldehyde, vanillin, coniferaldehyde, sinapaldehyde) which are of particular interest because they are aroma compounds and they can also interact with some wine compounds like anthocyanins and catechins, hence contributing to color changes (de Freitas et al. 2004, Pissarra et al. 2005, Nonier, Vivas 2007, Sousa et al. 2007, Sousa, Mateus 2010).

Indeed, one of these reactions most studied is the aldehyde-mediated association of anthocyanins and catechins through a Bayer acid-catalyzed condensation, yielding new pigments with different chromatic properties than the anthocyanin precursor (Timberlake and Bridle 1976, Rivas-Gonzalo et al. 1995, Dallas, Ricardo-da-Silva 1996, Escribano-Bailon, Dangles 1996, Fulcrand, Doco 1996, Es-Safi et al. 2000, Pissarra, Lourenco 2004, Sousa et al. 2007). More recently, cinnamic aldehydes have shown to react with catechin yielding a new class of brick-red catechin-pyrilium pigments (3-deoxyanthocyanidin derivatives), named oaklins (de Freitas et al. 2004). The formation of these compounds was confirmed in wine-like model solutions containing oak wood extract and an oaklin derived pigment was already found in a commercial table red wine (Sousa, Mateus 2005). Analogous compounds may be formed through the reaction of oak-derived aldehydes with proanthocyanidins. These latter are extracted from grapes to wine during winemaking and contribute to the astringency of red wines (Arnold, Noble 1980, Havsteen 1983, Haslam and Lilley 1988).

The present work deals with the formation of new oaklin pigments (see Fig. 4, structure **IX**) resulting from a direct reaction of a procyanidin dimer B4 ((+)-catechin-(4-8)-(-)-epicatechin) (**III**) with two cinnamic aldehydes (**I**), coniferaldehyde and sinapaldehyde. The newly formed compounds described herein for the first time point out the importance of procyanidins and cinnamic aldehydes in the formation of colored compounds during storage.

- MATERIALS AND METHODS

Samples. Coniferaldehyde and sinapaldehyde were purchased from Sigma-Aldrich®(Spain). Procyanidin B4 was obtained by hemisynthesis following the procedure described in the literature (Geissman and Yoshimur.Nn 1966).

Study of the reaction between procyanidin B4 and cinnamic aldehydes. Procyanidin B4 (1,7 mM, 2 mg) was incubated with coniferaldehyde (2,1 mM) and sinapaldehyde (2,1 mM) separately in 2 mL of 12% (v/v) hydroalcoholic solutions at pH 3.5 with a molar ratio of 1:1.2 (phloroglucinol:cinnamic aldehyde). These model solutions were kept at a temperature of 35 ºC and protected from light. The formation of new compounds was followed over time by HPLC-DAD using a reversed phase C-18 (Merck) column (250 x 4.6 mm i. d., particle size 5 µm), at 25 ºC. Solvents were (A) water/formic acid (95:5) and (B) acetonitrile. The elution gradient was performed using a L-2130 Merck pump from 10 to 35% B for 55 min at a flow rate of 1.5 $mL.min^{-1}$.

LC-MS conditions. Mass spectrometry analysis was performed using a Finnigan *SurVeyor* series liquid chromatograph, equipped with an API source, using an

electrospray ionization (ESI) probe. Solvents were (A) aqueous 0.1% acetic acid and (B) acetonitrile. The elution conditions were as follows: 0.5 $mL.min^{-1}$ flow rate; oven temperature, 35 ºC; elution began with linear gradient from 5 to 30% B in 40 min, from 30 to 40% in 10 min and from 40 to 100% in 5 min, followed by washing and re-equilibration of the column. The capillary voltage was 11 V, and the capillary temperature was 200 ºC. Spectra were recorded in positive ion mode between m/z 100 and 1200. The mass spectrometer was programmed to do a series of three scans: a full mass spectrum, a MS^2 spectrum of the most intense ion, and a MS^3 spectrum of the most intense ion in the second scan, using a relative collision energy of 45 V.

Synthesis and purification of the guaiacylcatechin-pyrylium-catechin (GCP-catechin) adduct. Procyanidin B4 (1,7 mM, 80 mg) was incubated with coniferaldehyde (8,3 mM) in 80 mL of a 12% (v/v) hydroalcoholic solution at pH 1 with a molar ratio of 1:4.8 (phloroglucinol:coniferaldehyde). Before the synthesis, several experiments at different pH values and molar ratios were tested in order to improve the yield of the reaction to a maximum of 9%. The model solution was kept at a temperature of 35 ºC and protected from light, and the formation of new compounds was followed by HPLC-DAD. When the reaction was completed, the sample was applied on a silica gel C-18 reversed phase SPE cartridge in order to remove inorganic salts and other impurities and the pigments were eluted with methanol acidulated with 2% HCl. Methanol was evaporated in a rotary evaporator at 38 ºC, and the sample was freeze-dried and stored at -18 ºC until use.

The sample was further applied into a 5 cm diameter medium-porosity sintered glass funnel with TSK Toyopearl gel HW-40(S) (Tosoh, Japan), connected to standard vacuum filtration glassware and gradually eluted with increasing percentages of acidified methanol (F1, 30%; F2, 40%; F3, 50%; F4, 60%; and F5, 80%). The criterion used for changing the percentages of methanol was the decrease in color intensity of the solution eluted from the column. The solvent of each fraction was partially evaporated in a rotary evaporator at 38 ºC, and the samples were freeze-dried and stored at -18 ºC until use.

Semi-preparative HPLC. Semi-preparative HPLC was performed in order to further isolate and purify the oaklin-catechin aducts, eluted in fraction F2 from toyopearl gel. This fraction was injected into a reversed phase C-18 (Merck) column (250 x 4.6 mm i. d., particle size 5 µm) at room temperature (volume injected was 1 mL). Solvents were (A) water/formic acid (95:5) and (B) acetonitrile. The elution gradient was performed using a L-2130 Elite LaChrom pump from 10 to 35% B for 65 min at a flow rate of 1.5 $mL.min^{-1}$ and detection was carried out at 500 nm using a L-2420 Elite LaChrom detector.

The sample collected from semi-preparative HPLC was further chromatographed on a Toyopearl HW40(S) column (30 cm x 1 cm) at a flow rate of 1 mL.min^{-1}, with an injection volume of 2.0 mL. The fractions obtained were gradually eluted with increasing percentages of acidified methanol from 30% to 100%. The solvent of each fraction was partially evaporated in a rotary evaporator at 38 ºC, and the samples were freeze-dried and stored at -18 ºC until use.

NMR measurements. In the NMR characterization of GCP-catechin adduct, ^{1}H NMR (500.13 MHz) and ^{13}C NMR (125.77 MHz) spectra were measured in DMSO:TFA (90:10) and on a Bruker Avance 500 spectrometer at 25 ºC with TMS as internal standard. ^{1}H assignments were made with the aid of 2D *g*COSY (^{1}H–^{1}H), whereas ^{13}C assignments were made on the basis of 2D *g*HSQC (^{1}H–^{13}C) and *g*HMBC experiments.

- RESULTS AND DISCUSSION

The reaction between cinnamic aldehydes and procyanidin B4 at pH 3.5 led to the formation of new compounds which showed maximum absorption in the visible range at λ_{max} 496 nm and at λ_{max} 506 nm from the reaction with coniferaldehyde and sinapaldehyde, respectively, indicating to have an orange/red color (Fig. 20).

The mass spectra and the respective MS^2 and MS^3 fragmentations of the new compounds formed in the reaction with coniferaldehyde ($[M]^+$ at m/z 737) and sinapaldehyde ($[M]^+$ at m/z 767) are presented in Fig. 21 and the respective fragmentation pattern in Fig. 22.

The molecular ions of these new compounds correspond to the sum of the molecular weights of procyanidin B4 (578 g.mol^{-1}) and the cinnamic aldehydes (coniferaldehyde and sinapaldehyde, 178 and 208, respectively) and the loss of a water molecule. Coniferaldehyde led to the formation of guaiacylcatechin-pyrylium-catechin adduct (GCP-catechin, 737 m/z), whereas sinapaldehyde led to the formation of syringylcatechin-pyrylium-catechin adduct (SCP-catechin, 767 m/z). Analysing in more detail the MS fragmentation (the products of the reaction of procyanidin B4 with coniferaldehyde) of GCP-catechin, the MS^2 spectrum shows a major fragment at m/z 585 ($[M-152]^+$), resulting from a retro Diels-Alder fragmentation (RDA), a fragment at m/z 719 corresponding to the loss of a water molecule ($[M-18]^+$), and two fragments corresponding to another RDA fragmentation ($[M-152-152]^+$ at m/z 433) and more two hydrogens ($[M-152-152+2H])^+$ at m/z 435). Further MS^3 fragmentation of the ion at m/z 585 yielded two fragments at m/z 433 and m/z 435. The other pigment formed in the reaction of procyanidin B4 with sinapaldehyde followed a similar fragmentation scheme.

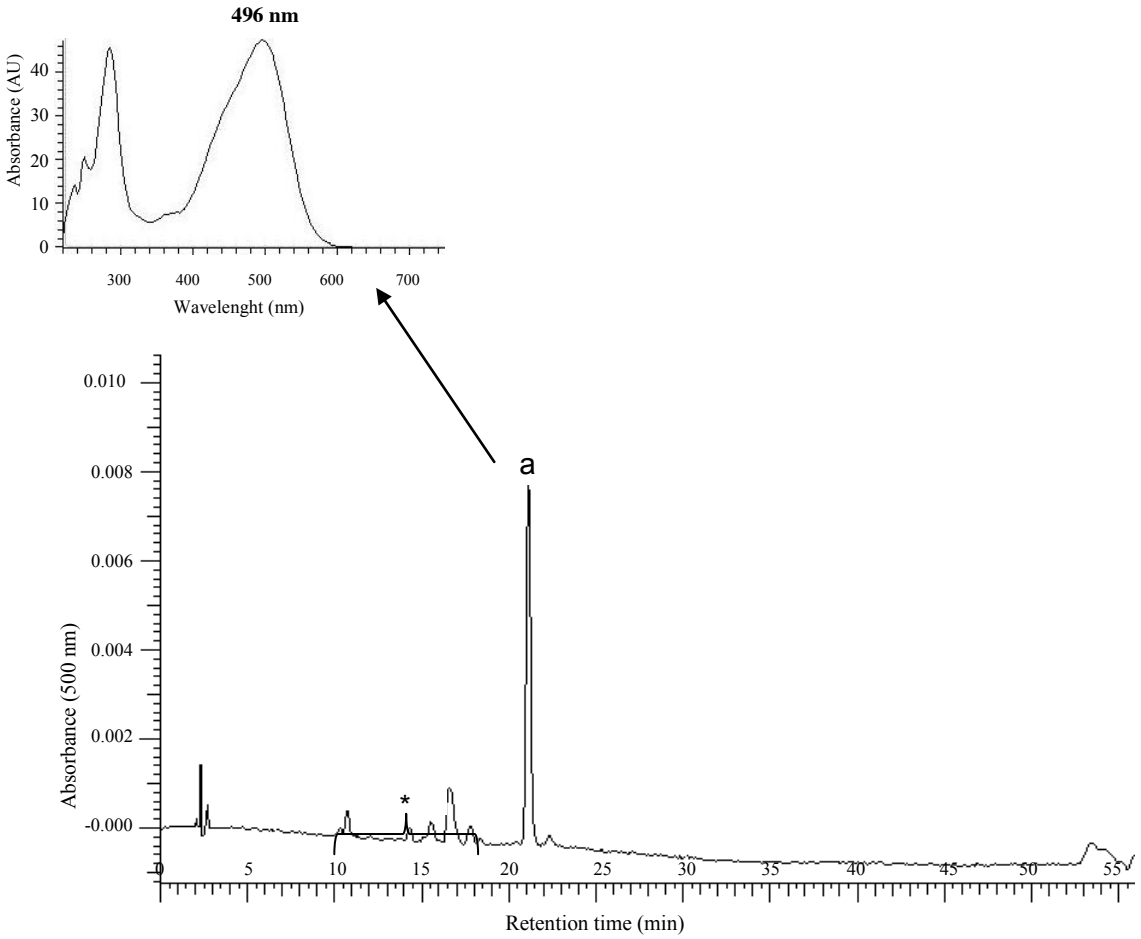

Fig. 20 - HPLC chromatogram recorded at 500 nm of the model solution containing B4 and coniferaldehyde, after 5 days of reaction at pH 3.5 and 35ºC. UV/Vis spectrum of GCP-catechin (* - unidentified peaks; a - GCP-catechin).

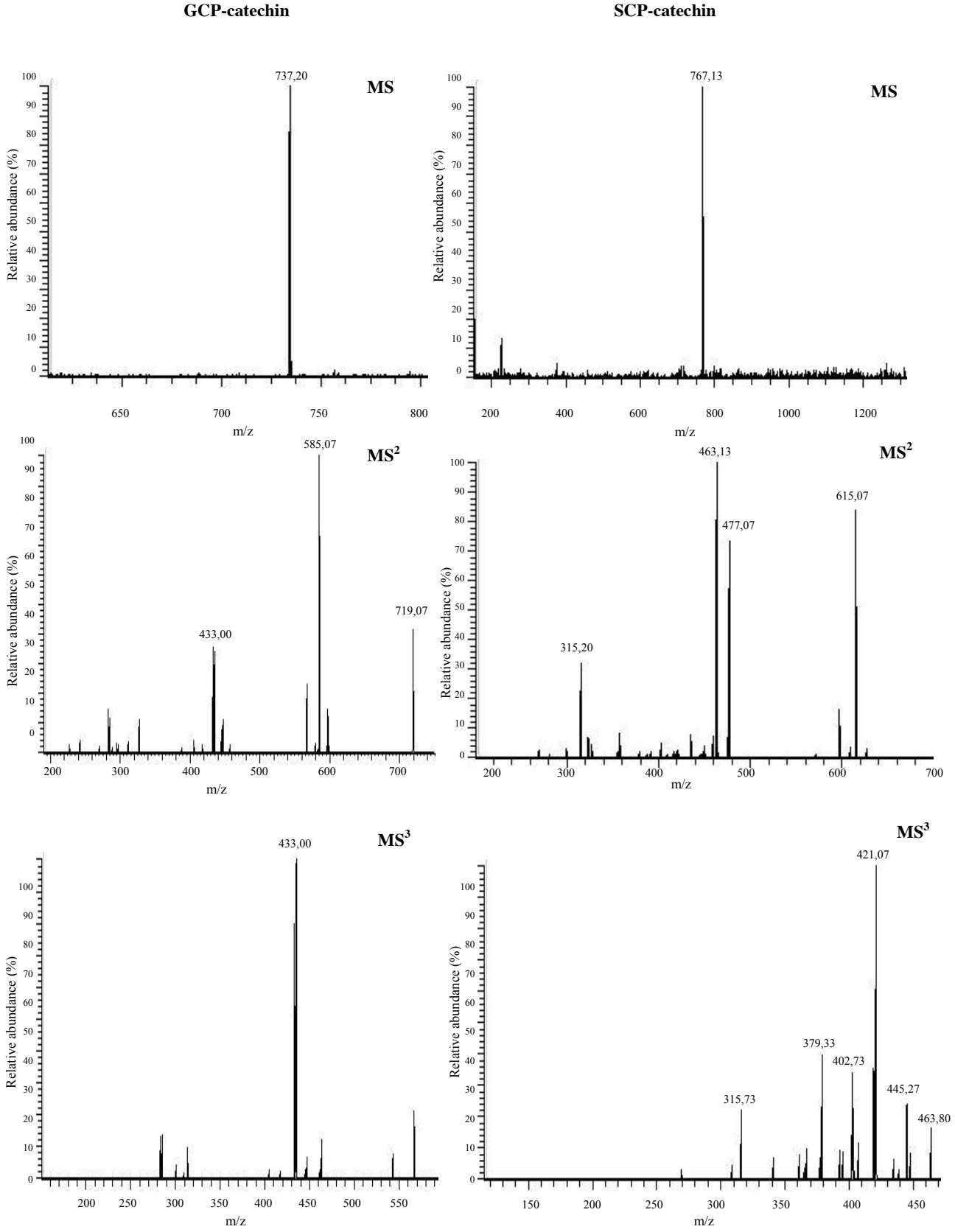

Fig. 21 - Mass spectra and respective MS^2 (of the molecular ion) and MS^3 (of the main fragment in MS^2) fragmentations for GCP-catechin and SCP-catechin adducts.

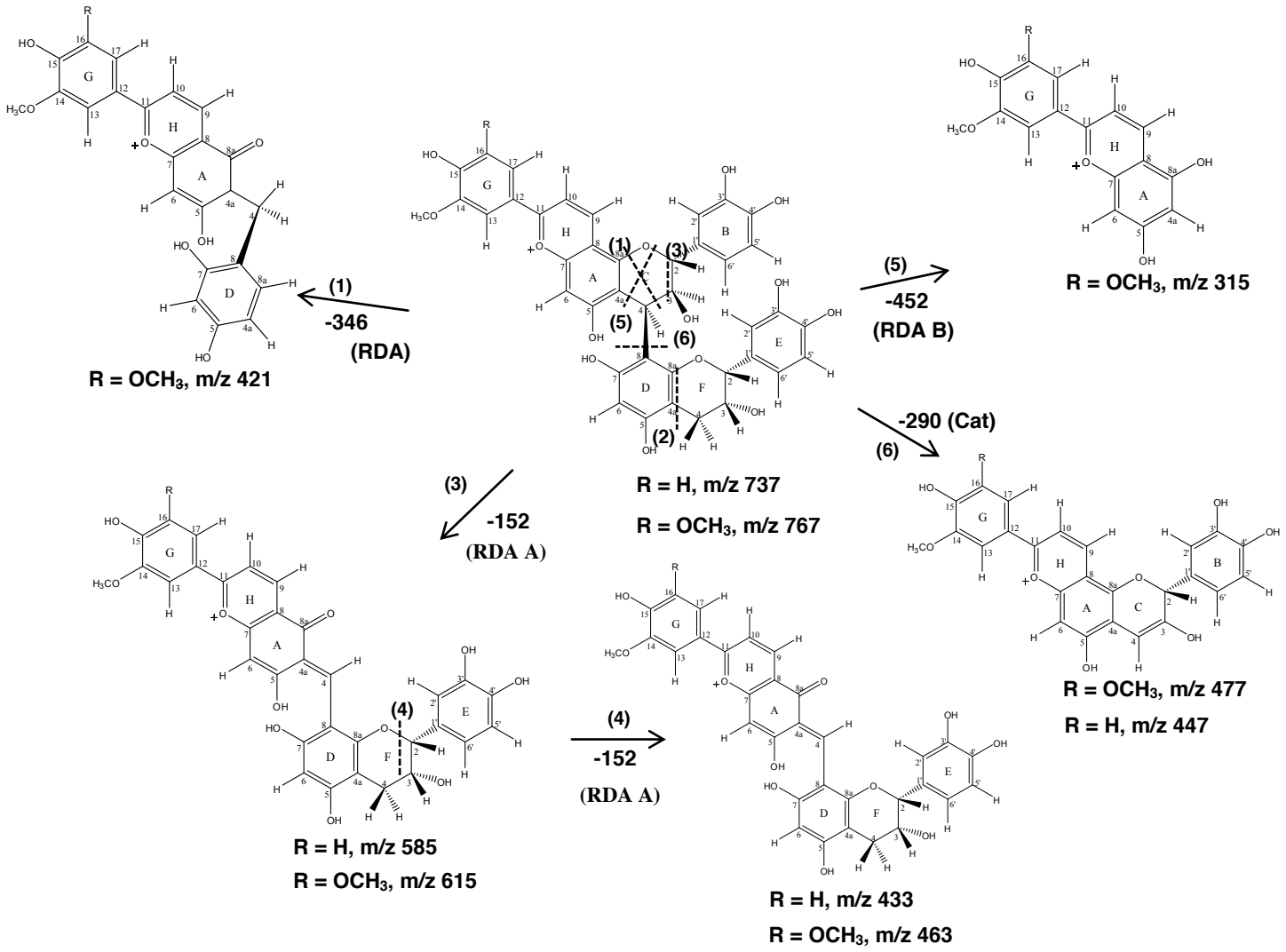

Fig. 22 – Fragmentation pattern of GCP-catechin (R = H) and SCP-catechin (R = OCH_3) adducts in the positive ion mode.

Synthesis and structural characterization of GCP-catechin adduct. The synthesis reaction was carried out at pH 1.0 and the sample collected from semi-preparative HPLC. In these conditions, three pigments were formed and co-eluted in HPLC at 21 minutes and were only detected after further purification by Toyopearl gel chromatography. These pigments were analyzed separately by LC-MS and correspond to two compounds with the same molecular weight (GCP-catechin isomers) and another one with a molecular weight corresponding to a GCP-catechin-catechin adduct ($[M]^+$ at m/z 1025). GCP-catechin isomers should correspond to positional isomers, as there are no chiral centers in their structure.

The GCP-catechin-catechin structure was not detected in the reaction at pH 3.5. This structure should only be formed at lower pH because procyanidin B4 may be

decomposed through its interflavonoid linkage yielding catechin monomers in solution that may react by acid-catalyzed condensations with a molecule of procyanidin dimer B4 forming procyanidin dimer-catechin adducts. Moreover, a GCP monomer structure was also detected in the mass spectra ($[M]^+$ at m/z 449) in the reaction at pH 1.0 as it is expected to be formed due to the presence of catechin monomers in solution that may react with the cinnamic aldehydes, like the mechanism described in the literature for the formation of these pigments (*10*). However, only the major GCP-catechin isomer was obtained in enough quantity to be characterized by NMR.

The ^{1}H chemical shifts were assigned using 1D and 2D NMR techniques (*g*COSY), and the assignment of the carbon resonances was made using 2D techniques (*g*HSQC and *g*HMBC techniques) (Table 1).

The new vinylic protons 9H and 10H revealed a clear correlation in the COSY spectrum and were attributed to the two doublets located at δ 9.00 and 8.30ppm, respectively. The assignment of the carbon resonances was possible using two-dimensional NMR techniques (HSQC and HMBC). Carbons 9H and 10H were assigned at δ 146.9 and 111.3ppm through HSQC correlation with the respective protons.

Concerning the procyanidin B4 moiety, the proton 4C was assigned to two different signals, δ 3.74 and 3.84ppm, H-4C$_1$ and H-4C$_2$, respectively, through the characteristic ABMX spin system of the pyran ring C observed in the COSY spectrum. Proton 3C was also assigned to two different signals, δ 3.52 and 3.50ppm, H-3C$_1$ and H-3C$_2$, respectively, from its correlations with proton 4C. These two signals may be attributed to two rotamers resulting from a rotation throughout the interflavonoid C-4C-C-8D linkage characterized by different C3-C4-C8-C2 dihedral angles (Fletcher, Porter 1977). Only protons from pyranic rings F and C show different chemical shifts for the two rotamers probably because their chemical environment is the most affected by the C-4C-C-8D linkage rotation.

Proton H-4αF was assigned to two different signals, δ 2.86 and 2.91ppm, H-4αF$_1$ and H-4αF$_2$, respectively, through the characteristic ABMX spin system of the pyran ring C observed in the COSY spectrum. The same occurs to proton H-4βF with two different signals, δ 3.44 and 3.49ppm, H-4βF$_1$ and H-4βF$_2$, respectively. Proton H-3F was also assigned to two different signals, δ 3.52 and 3.52ppm, H-3F$_1$ and H-3F$_2$, respectively, from its correlations with protons H-4αF(weak) and H-4βF(strong). H-3F also correlates with H-2F, which also shows two different signals, δ 5.20 and 5.30 ppm, H-2F$_1$ and H-2F$_2$, respectively. The only proton detected on ring D (H-6D) was assigned to the singlet at δ 7.02ppm.

Table 1. ^{1}H and ^{13}C NMR data and HMBC and HSQC correlations of isomer **1** of GCP-catechin, determined in DMSO/TFA (90:10).

Position	**δ ^{1}H (ppm); *J* (Hz)**	**δ ^{13}C (ppm)**	**HMBC**	**HSQC**
Ring G				
12G		120.3	H-16G	
13G	7.92; s	112.4		H-13G
14G		149.0	H-16G, H-13G, OMe	
15G		156.0	H-13G, H-16G	
16G	7.09*	117.5		H-16G
17G	8.08; d	126.0		H-17G
OMe	3.96; s	56.2		OCH3
Ring H				
9H	9.00; d	146.9		H-9H
10H	8.30; d	111.3		H-10H
11H		170.2	H-10H, H-13G	
Ring A, B, C				
2C	na	na		H-2C
$3C_1$	3.52*	60.4		$H\text{-}3C_1$
$3C_2$	3.50*			$H\text{-}3C_2$
$4C_1$	3.74*	65.5		$H\text{-}4C_1$
$4C_2$	3.84*			$H\text{-}4C_2$
4aA		110.5	H-6A	
5A		168.8	H-6A	
6A	7.17; s	95.4		H-6A
7A		156.9	H-6A	
8A		111.5	H-6A, H-10H	
8aA		153.7	H-9H	
1'B		129.6	H-2'B	
2'B	6.76*	118.0		H-2'B
3'B		145.3	H-2'B	
4'B		145.5	H-6'B	
5'B	6.77*	115.4		H-5'B
6'B	6.81*	118.4		H-6'B

Ring D, E, F

$2F_1$	5.20*	80.2		H-$2F_1$
$2F_2$	5.30*			H-$2F_2$
$3F_1$	4.85*	66.3		H-3αF
$3F_2$	4.26*			H-3βF
$4\alpha F_1$	2.86*			H-$4\alpha F_1$
$4\beta F_1$	2.92*	40.0		H-$4\beta F_1$
$4\alpha F_2$	3.44*			H-$4\alpha F_2$
$4\beta F_2$	3.49*			H-$4\beta F_2$
4aF		na		
5D		145.0	H-6D	
6D	7.02; s	115.5		H-6D
7D		145.0	H-6D	
8D		na		
8aD		na		
1'E		128.4	H-2F, H-6'E	
2'E	6.88*	115.0		H-2'G
3'E		144.8	H-2'E	
4'E		145.0	H-6'E	
5'E	6.88*	118.5		H-5'G
6'E	6.74*	115.4		H-6'G

na - not attributed; s - singlet; d - doublet; brs - broad singlet; * - unresolved

Carbons C-4aA and C-7A were determined from their long range $^{1}H–\ ^{13}C$ correlation with H-6A. The quaternary carbons C-5A and C-8aA were assigned at *δ* 168.8 and 153.7ppm from their long distance correlations with protons H-6A and H-9H, respectively, observed in the HMBC spectrum.

These correlations as well as the lack of a long-distance correlation between carbon C-8aA and the singlet at 7.17ppm (H-6A) identify unambiguously the position of the pyrylium ring H linkages onto carbons C-7A and C-8A, as they could not be observed if ring H was formed between the hydroxyl group at carbon C-5A and the carbon C-6A.

The chemical shift of the remaining protons and carbons identified in Table 1 were easily established by HSQC and HMBC techniques.

Formation Mechanism. The hypothetic mechanism of formation of these new pigments from the reactions between procyanidin B4 and cinnamic aldehydes is represented in Fig. 23 - Hypothetical mechanism for the formation of oaklin-catechins **IX** obtained from the reaction between procyanidin B4 **III** and cinnamic aldehydes **I**. By analogy with the mechanism described in the literature for the formation of monomeric

oaklins, the reaction starts with the protonation of the cinnamic aldehyde **I** in acidic medium, forming a carbocation in the carbonyl carbon **II**, followed by a nucleophilic attack of ring A of procyanidin B4 **III**, leading to structure **IV**. The A ring attack may occur from position C6 or preferentially from position C8. Indeed, the negative formal charge in ring A is expected to be higher at carbon 8, as it is well documented for flavylium compounds in the reaction leading to the formation of catechin-alkyl/aryl-anthocyanin adducts (Bendz et al. 1967, Rivas-Gonzalo et al. 1995). The putative presence of compound **IV** was evidenced in the mass spectra by the presence of a protonated molecular ion ($[M+1]^+$) at m/z 757. The dehydration of the resulting protonated adduct **V** yields a new carbocation **VI**, which undergoes a rearrangement leading to carbocation **VII**. This carbocation suffers an intra-molecular nucleophilic attack by the hydroxyl group at carbon 7 of ring A, leading to structure **VIII**. The putative presence of compound **VIII** was evidenced in the mass spectra by the presence of a protonated molecular ion ($[M+1]^+$) at m/z 739. The final oxidation yields the structure **IX,** which has the pyrylium ring H associated with the aromatic ring A and constitutes a chromophore group. The extended conjugation of the π electrons are at the origin of their maximum absorption around 500nm. The GCP-catechin position isomer formed at pH 1.0 in lower amount may result from an initial nucleophilick attack from position C6 to the aldehyde resulting in the formation of a new pyranic ring involving position 5 and 6 of ring A.

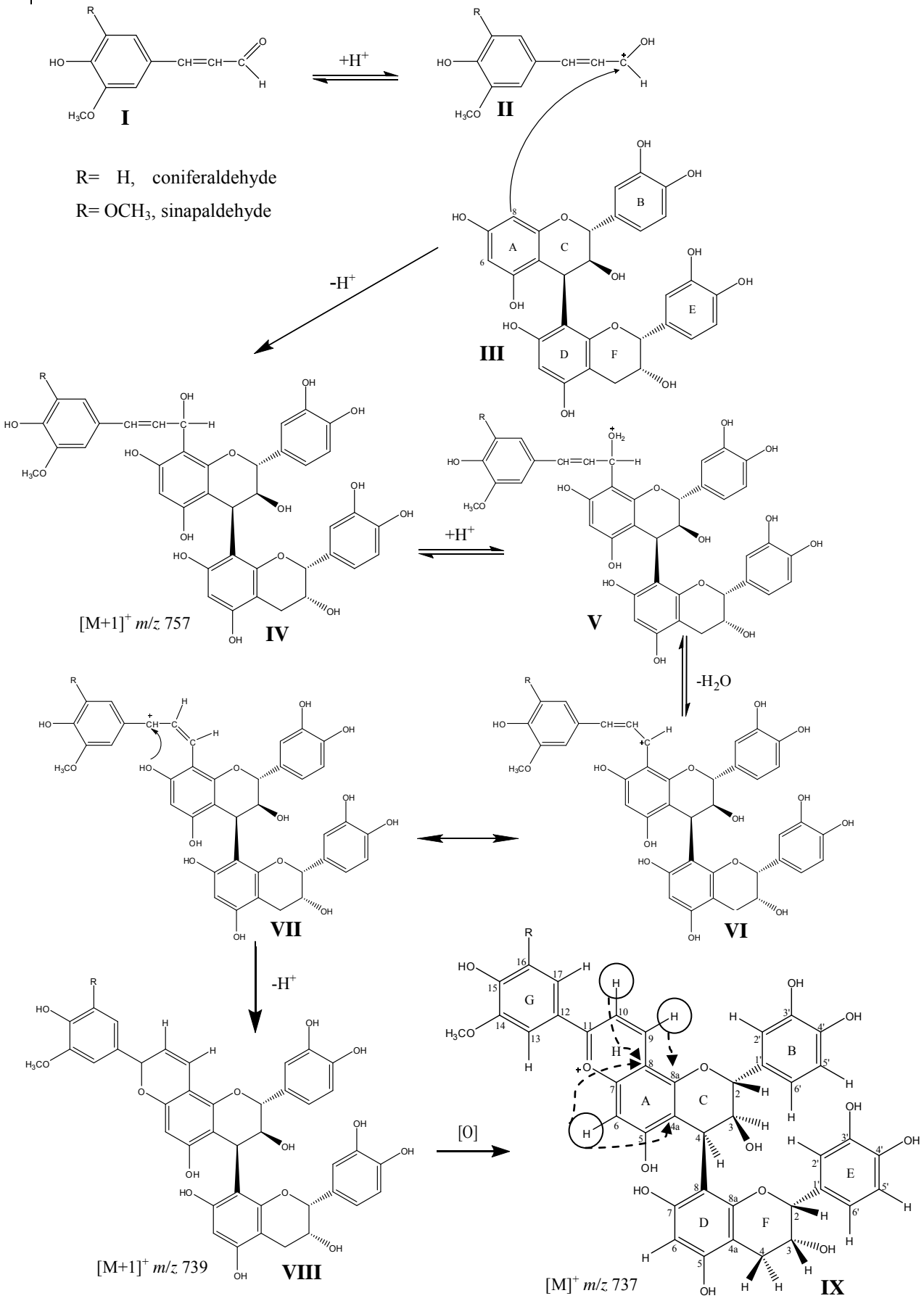

Fig. 23 - Hypothetical mechanism for the formation of oaklin-catechins **IX** obtained from the reaction between procyanidin B4 **III** and cinnamic aldehydes **I**.

- CONCLUSION

Procyanidin B4 showed ability to react directly with cinnamic aldehydes, namely coniferaldehyde and sinapaldehyde to give orange pigments (oaklins). Oaklins monomers formed from the reaction between catechin and cinnamic aldehydes have already been detected in wine model solutions (11-guaiacylcatechinpyrylium) and wine (Sousa et al. 2005). Therefore, attending to the relative high amount of procyanidin dimers in real wines it is also expected that oaklin-catechin adducts may also occur in wine and even play a role in some color changes observed during the aging process. Nonetheless, further studies are still required to unequivocally detect the formation of oaklin-catechins in wine and to understand their overall contribution to wine properties, such as color and taste.

- AKNOWLEDGEMENTS

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Chapter 2

A novel reaction mechanism for the formation of deoxyanthocyanidins

Sousa, A., Mateus, N., de Freitas, V.

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In this work, the first author was responsible for undertaking all experimental activities with the assistance of MS and NMR technicians and guided by the scientific knowledge of the rest of the authors.

A novel reaction mechanism for the formation of deoxyanthocyanidins

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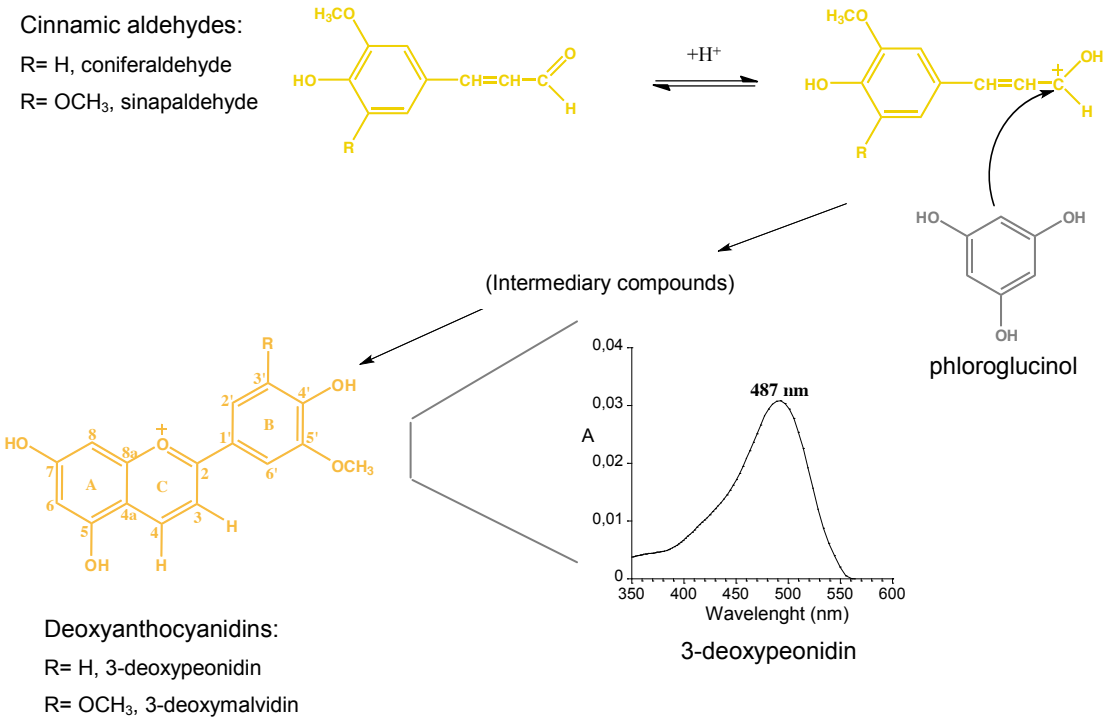
The synthesis of deoxyanthocyanidins from the reaction between cinnamic aldehydes (coniferaldehyde and sinapaldehyde) and phloroglucinol is reported herein. The resulting structures were characterised by visible, MS and NMR spectroscopy.



KEYWORDS: deoxyanthocyanidins; aldehydes; phloroglucinol; flavonoids; NMR; mass spectrometry

- INTRODUCTION

Deoxyanthocyanidins are yellowish pigments found in several food plants such as corn, black tea leaves and sorghum. Sorghum is one of the most important cereal crops in the world and is rich in 3-deoxyanthocyanidins, particularly luteolinidin and apigeninidin (Coggon, Moss 1973, Sweeny and Iacobucci 1977, 1981). Deoxyanthocyanidins are considered the chemical ancestors of anthocyanins, the ubiquitous water-soluble pigments that are found in flowers and fruits and are responsible for their impressive blue and purple colors (Sweeny and Iacobucci 1977).

Open chalcone forms of the common anthocyanins are assumed to be crucial in reactions leading to irreversible degradation of anthocyanins, particularly under weakly acidic to weakly alkaline solution conditions (Mazza and Brouillard 1987, Francis 1989, Cabrita et al. 2000, Torskangerpoll and Andersen 2005, Sadilova et al. 2007). However, the natural yellow deoxyanthocyanidins are much more stable in slightly acidic solutions than anthocyanins and anthocyanidins, which points to the potential advantage of this type of compounds as viable commercial food colourants, and justifies the research developed in the chemistry of 3-deoxyanthocyanins and, in particular, the search for new colourants with significant stability (Iacobucci and Sweeny 1983, Dangles and Elhajji 1994, Khalil et al. 2010). In addition, more studies have demonstrated other potential applications for these compounds, such as their use as hair dyes, laser dyes, sensitizers for solar cells and molecular-level memory systems (Roque et al. 2002).

Despite the interest in these types of compounds, few synthetic approaches have been made towards deoxyanthocyanidins and the procedures described in the literature are complex (Pratt and Robinson 1922, 1923, Pratt et al. 1924, Pratt and Robinson 1925, Sweeny and Iacobucci 1977, Kuhnert et al. 2001). Only in recent years attemps have been made to synthesize these compounds with simpler methods (Mas 2003, Chassaing et al. 2007, Kueny-Stotz et al. 2007).

- MATERIALS AND METHODS

The synthesis of 3-deoxyanthocyanidins **9** (Fig. 24) from the reaction of phloroglucinol **3** with two cinnamic aldehydes **1** (Fig. 27), coniferaldehyde and sinapaldehyde, is described herein and their chemical structure and mechanism of formation elucidated.

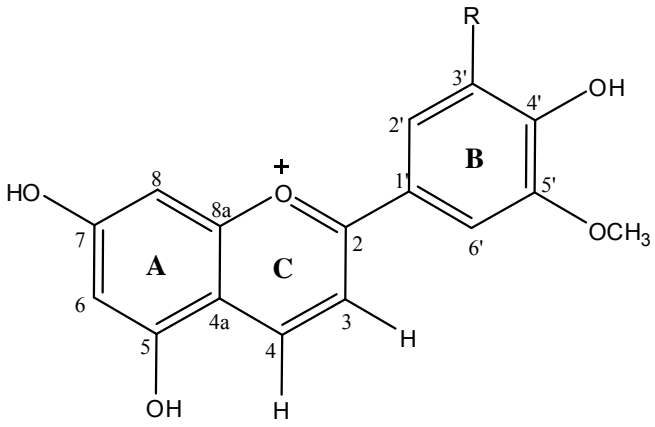


Fig. 24 - Structure of the new synthesized 3-deoxyanthocyanidins **9** (R=H, 3-deoxypeonidin; R=OCH_3, 3-deoxymalvidin).

Phloroglucinol **3** (8mM) was incubated with coniferaldehyde and sinapaldehyde **1** separately under different conditions of pH, percentage of ethanol in water and molar ratios. These model solutions were kept at a temperature of 35 ºC and protected from light. The formation of new compounds was followed over time by HPLC-DAD using a reversed phase C-18 (Merck) column (250 x 4.6 mm i. d., particle size 5 µm) at 25 ºC. Solvents were (A) water/formic acid (95:5) and (B) acetonitrile. The elution gradient was performed using a L-2130 Merck pump from 10 to 35% B for 55 min at a flow rate of 1.5 $mL.min^{-1}$.

- RESULTS AND DISCUSSION

The mass spectra of these compounds obtained by LC–DAD/ESI/MS in the positive ion mode showed a molecular ion $[M]^+$ at m/z 285 (3-deoxypeonidin) through the reaction with coniferaldehyde, and $[M]^+$ at m/z 315 (3-deoxymalvidin), from the reaction with sinapaldehyde. In addition, the MS^2 spectrum of 3-deoxipeonidin shows a major fragment at m/z 270 (loss of a methyl group, $[M-15]^+$), and further MS^3 fragmentation of the ion at m/z 270 yielded a fragment at m/z 242 (loss of CO, $[M-28]^+$). The other pigment formed in the reaction of phloroglucinol with sinapaldehyde followed a similar fragmentation scheme.

The two compounds, 3-deoxypeonidin and 3-deoxymalvidin revealed a maximum absorption in the visible spectrum at 487 nm and at 492 nm (Fig. 25), respectively, conferring them a yellow colour.

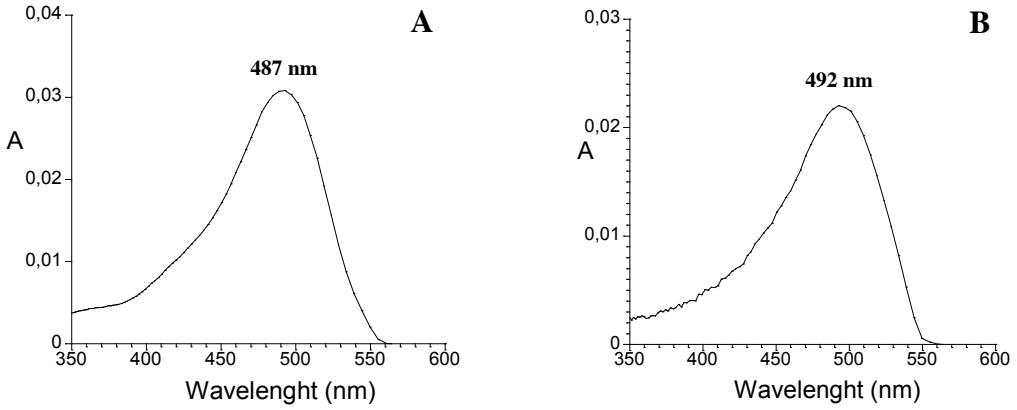


Fig. 25 - Visible spectra of 3-deoxypeonidin (**A**) and 3-deoxymalvidin (**B**) determined directly by HPLC-DAD (pH≈2).

The kinetics of formation of 3-deoxypeonidin from the reaction between phloroglucinol and coniferaldehyde were studied at different conditions of pH, percentage of ethanol in water and molar ratios (Fig. 26). It can be seen from Fig. 26A that the reaction was faster and with a better yield (8%) at a molar ratio of 1:10 (phloroglucinol:aldehyde) for a constant value of pH 1.5 and 12% ethanol. At this molar ratio (1:10, phloroglucinol:aldehyde), the reaction performed at pH 1.0 and 12% ethanol was faster (the maximum yield was achieved in 4 days) and yielded larger amounts of the new pigment compared with pH 1.5 and higher percentages of ethanol. However the yield obtained by this method (8%) is lower than the yield obtained with the methods described in the literature for the preparation of other deoxyanthocyanidins (Pratt and Robinson 1922, 1923, Pratt et al. 1924, Pratt and Robinson 1925, Sweeny and Iacobucci 1977, Kuhnert et al. 2001, Mas 2003, Chassaing et al. 2007, Kueny-Stotz et al. 2007).

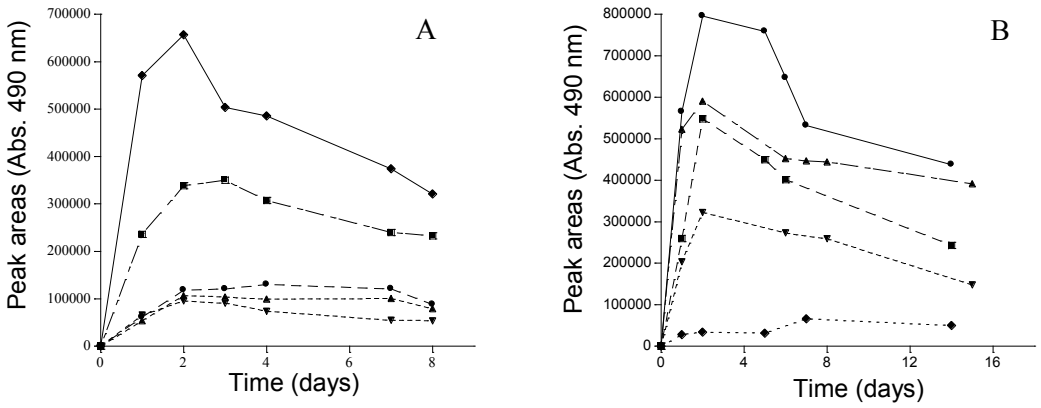


Fig. 26 - Kinetic study of the reaction between phloroglucinol and coniferaldehyde. A – Influence of molar ratios (phloroglucinol:coniferaldehyde) at pH 1.5, 12% ethanol: (●) 1:1; (■) 1:5; (♦) 1:10; (▲) 5:1; (▼) 10:1; B – Influence of diferent pH values and percentage of ethanol: (●) pH 1.0 /12% ethanol ; (■) pH 1.5 /12% ethanol; (♦) pH 3.5 /12% ethanol; (▲) pH 1.0/20% ethanol; (▼) pH 1.5/30 % ethanol (for a molar ratio of 1:10, phloroglucinol:coniferaldehyde).

3-deoxypeonidin was the only deoxyanthocyanidin synthesized in large amounts and its structure was fully elucidated by ^{1}H and ^{13}C NMR spectroscopy using 2D techniques (COSY, HSQC, HMBC) (Table 2).

Concerning the pyrylium ring C, the new vinylic protons 3C and 4C revealed a clear correlation in the COSY spectrum and were attributed to the two doublets located at *δ* 8.26 (*J* = 8,9 Hz) and 9.05ppm (*J* = 8,7 Hz), respectively. Carbons 3C and 4C were assigned at *δ* 110.5 and 148.5ppm through HSQC correlation with the respective protons. Carbon 2C was assigned at *δ* 170.6 from its long distance correlations observed in the HMBC spectrum.

The protons 2'B, 5'B were assigned to the doublets at *δ* 7.91 (*J* = 2.0 Hz) and 7.10 ppm (*J* = 8.6 Hz), respectively and the proton 6'B to the double doublet at *δ* 8.06 ppm. The methoxyl group from ring B was attributed to the singlet at *δ* 3.96 ppm.

Protons 6A and 8A revealed a clear correlation in the COSY spectrum and were attributed to the singlets at *δ* 7.03 and 6.76ppm, respectively. Carbons 6A and 8A were assigned at *δ* 95.4 and 102.3ppm, respectively through HSQC correlation with the respective protons. Carbons 5A and 7A were assigned at *δ* 158.5 and 170.7ppm from their long distance correlations with protons 6A, 4C and 8A, 6A respectively, observed in the HMBC spectrum. The carbons 4aA and 8aA were assigned at *δ* 112.5 and 158.9ppm from their long distance correlations with protons 8A, 3C and 8A, 4C, 6A respectively, observed in the HMBC spectrum.

These correlations identify unambiguously the position of the aromatic ring A linked to the pyrylium ring C by carbons 8aA and 4aA.

The chemical shift of the remaining protons and carbons identified in Table 2 were easily established by HSQC and HMBC techniques.

Table 2. ^{1}H and ^{13}C NMR data and HMBC and HSQC correlations of 3-deoxypeonidin, determined in DMSO/TFA (90:10).

Position	**δ ^{1}H (ppm); *J* (Hz)**	**δ ^{13}C (ppm)**	**HMBC**	**HSQC**
Ring A				
5A		158.5	H-6A, H-9C	
6A	7,03; s	95.4		H-6A
7A		170.7	H-6A, H-8A	
8A	6,76;s	102.3		H-8A
4aA		112.5	H-8A	
8aA		158.9	H-6A, H-8A	
Ring B				
1′B		120.2	H-6′B, H-5′B, H-3C, H-4C	H-1′B
2′B	7.91; d, 2.0	112.4		
3′B	3.96; s	149.2	H-2′B, H-6′B, H-5′B, OCH_3	OCH_3
4′B		155.8	H-2′B, H-6′B, H-5′B	H-4′B
5′B	7,10; d, 8,6	117.1		
6′B	8,06; dd, 2.0	125.6		
Ring C				
2C		170.6	H-4C, H-3C, H-6′B, H-2′B, H-5′B, H-8A	
3C	8.26; d, 8,9	110.5		H-3C
4C	9.05; d, 8,7	148.5		H-3C

s - singlet; d - doublet; dd – double doublet

The hypothetic mechanism of formation of these pigments from the reaction between phloroglucinol and cinnamic aldehydes is represented in Fig. 27.

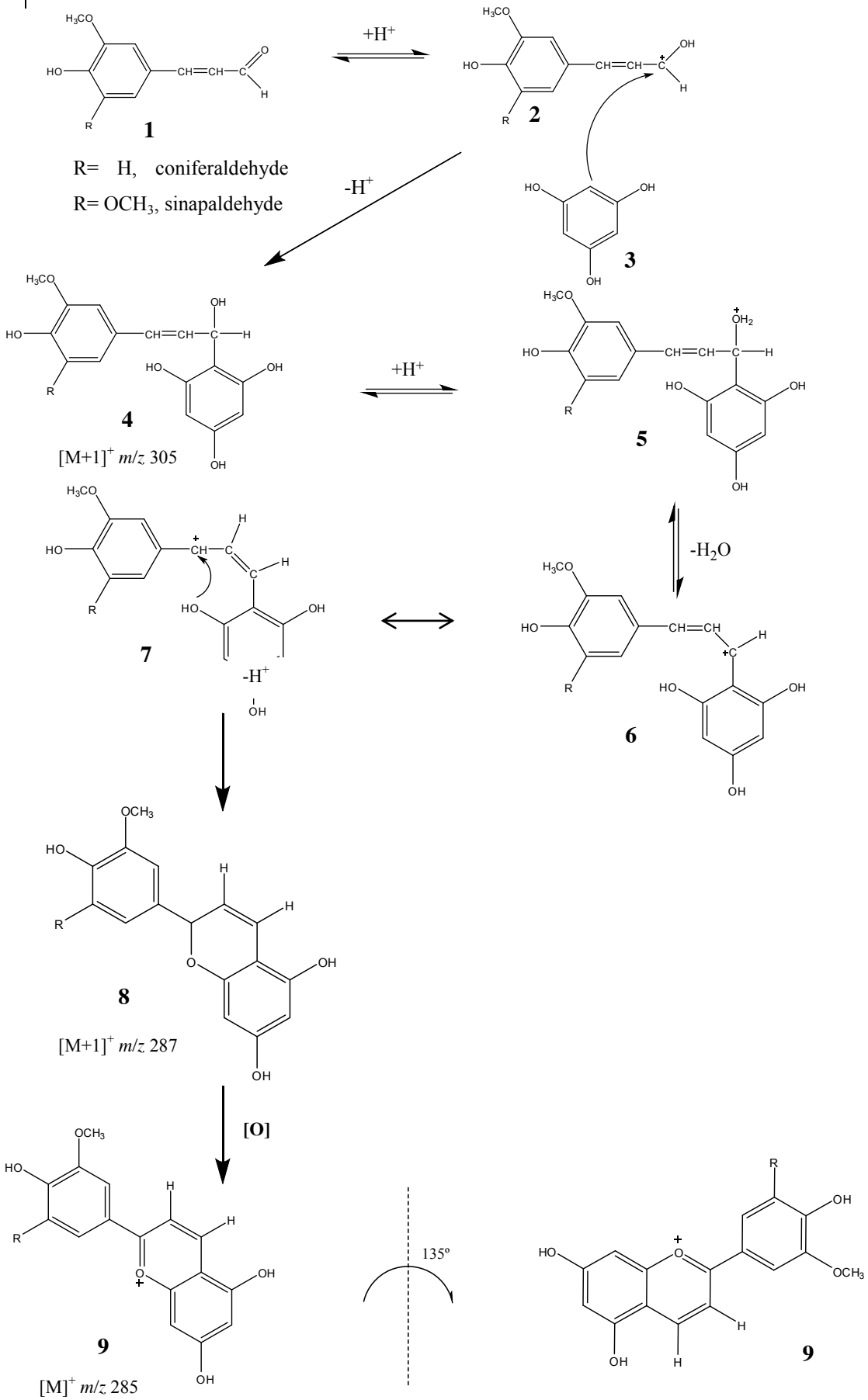


Fig. 27 - Mechanism proposed for the formation of 3-deoxypeonidin and 3-deoxymalvidin **9** obtained from the reaction between phloroglucinol **3** and cinnamic aldehydes **1**.

The reaction starts with the protonation of the cinnamic aldehyde **1** in acidic medium, forming a carbocation in the carbonyl carbon **2**, followed by a nucleophilic attack of phloroglucinol **3**, leading to structure **4**. The putative presence of compound **4** was evidenced by ESI-MS direct analysis of the reaction solution showing a protonated molecular ion $[M+1]^+$ at m/z 305, with a fragmentation scheme in MS^2 ($[M+1-18]^+$, $[M+1-126]^+$, $[M+1-124]^+$) and MS^3 ($[M+1-124-28]^+$, $[M+1-124-18]^+$) spectra. The dehydration of the resulting protonated adduct **5** yields a new carbocation **6**, which undergoes a rearrangement leading to carbocation **7**. This carbocation suffers an intra-molecular nucleophilic attack by the hydroxyl group at the adjacent carbon of phloroglucinol, leading to structure **8**. The putative presence of compound **8** was also confirmed in the ESI-MS spectra which showed a protonated molecular ion $[M+1]^+$ at m/z 287, with a fragmentation scheme in MS^2 ($[M+1-32]^+$, $[M+1-126]^+$) spectra. The final oxidation yields the structure **9** which has the new pyrylium ring C associated with the aromatic ring A and constitutes a chromophore group. The resulting structure is a 3-deoxyanthocyanidin and the extended conjugation of the π electrons is at the origin of their maximum absorption around 490 nm, which represents a hypsochromic shift from their anthocyanidin relatives (Stevenso.Pe 1965).

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Chapter 3

Thermodynamics, Kinetics and Photochromism of Oaklins: a Recent Family of Deoxyanthocyanidins

Sousa, A., Petrov, V., Araújo, P., Mateus, N., Pina, F., de Freitas, V.

J. Phys. Chem. B. 2013, 117(6), 1901-10

In this work, the first author actively participated in all of the experimental activities. He counted with the help of Paula Araújo in the chemical synthesis of the tested compounds and was instructed and guided by Vesselin Petrov in the pH jumps and photochromic experiments as well as in the model data treatment. He was always actively supported by the scientific expertise of the rest of the authors.

Thermodynamics, Kinetics and Photochromism of Oaklins: a Recent Family of Deoxyanthocyanidins.

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Two oaklins Guaiacylcatechinpyrylium (GCP) and Syringylcatechinpyrylium (SCP) and a model compound Deoxypeonidin (DOP) were synthesized and the rate and equilibrium constants of the respective pH dependent network of chemical reactions were calculated. In contrast to anthocyanins, the three compounds possess a small *cis-trans* isomerization barrier and hence the rate of the *trans*-chalcone formation follows a bell shaped curve as a function of pH. The three compounds exhibit photochromism obtained by irradiation of the *trans*-chalcone, which, depending on pH, leads to the colored species flavylium cation and quinoidal base. The flash photolysis together with pH jumps followed by UV-Vis absorption and stopped flow is a very useful tool to achieve the rate and equilibrium constants of the network of chemical reactions followed by these molecules. Oaklin compounds which are formed in wine aged in oak barrels present physical-chemical properties more similar to simpler deoxyanthocyanidins rather than anthocyanins and may play a significant role in color changes observed in wine aging. Given their higher stability, they may be regarded as potential food colorants.

KEYWORDS: oaklins, deoxyanthocyanidins, anthocyanins, flavylium network, photochemistry, food colorants, wine aging

▪ INTRODUCTION

2-phenyl-1-benzopyrilium (flavylium) derivatives, which comprise anthocyanins, deoxyanthocyanins, anthocyanidins as well as bio-inspired synthetic flavylium compounds, have been a recurrent subject of research through more than a century (Pina, Melo 2012). This is due to the role played by anthocyanins as colorants of most

flowers and fruits and to the antioxidant properties of these compounds and the consequent potential of their applications in human health (Kumpulainen 1996).

In general, independently of their natural or synthetic origin, flavylium derivatives follow the same network of chemical reactions as reported on Fig. 28 for malvidin-3-glucoside.

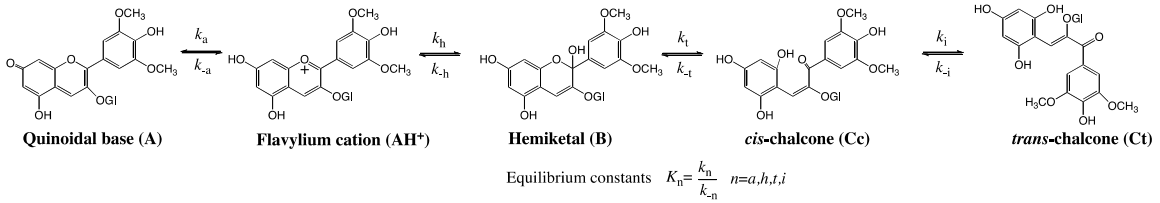

Fig. 28 - Network of chemical reactions of malvidin-3-glucoside.

Despite the apparent complexity of the pH dependent chemistry of flavylium compounds, due to the manifold of chemical reactions involving these compounds, the system can be viewed as a single acid-base equilibrium involving flavylium cation and its conjugate base **CB**, eq.(1)

$$\mathbf{AH^+} + H_2O \rightleftharpoons \mathbf{CB} + H_3O^+ \qquad K'_a = K_a + K_h + K_h K_t + K_h K_t K_i \qquad (1)$$

CB, defined as the sum of the concentrations of the other species in the network, [CB]=[A]+[B]+[Cc]+[Ct] (Brouillard and Delaporte 1977, Brouillard and Dubois 1977, Brouillard, Delaporte 1978, Brouillard and Lang 1990, Pina 1998).

The global equilibrium defined by eq.(1) is decomposed in their components according to eq.(2) to eq.(5).

$\mathbf{A}H^+ + H_2O \rightleftharpoons \mathbf{A} + H_3O^+$	K_a	Proton transfer	(2)
$\mathbf{A}H^+ + 2H_2O \rightleftharpoons \mathbf{B} + H_3O^+$	K_h	Hydration	(3)
$\mathbf{B} \rightleftharpoons \mathbf{Cc}$	K_t	Tautomerization	(4)
$\mathbf{Cc} \rightleftharpoons \mathbf{Ct}$	K_i	Isomerization	(5)

The percentage of each of the “basic species of CB” is dramatically dependent on the substitution pattern of the flavylium core. For example in anthocyanins **B** is the major

species, while in 7,4′-dihydroxyflavylium it is the *trans*-chalcone **Ct** (Pina, Melo 1998, Pina et al. 2012). It is worth noting the reversibility of all the reactions, at least in acidic medium, which makes the flavylium network a unique multi-component system. Photochemistry takes place upon irradiation of **Ct** leading to **Cc**, which spontaneously gives flavylium cation or/and quinoidal base depending on pH. The photochemical system is reversible and **Ct** is recovered through the thermal back reaction.

In this work, the compounds Guaiacylcathechinpyrylium (GCP), syringylcatechinpyrylium (SCP) and the model compound deoxypeonidin (DOP) (Fig. 29) were synthesized and the thermodynamic and the kinetics of the respective network fully described.

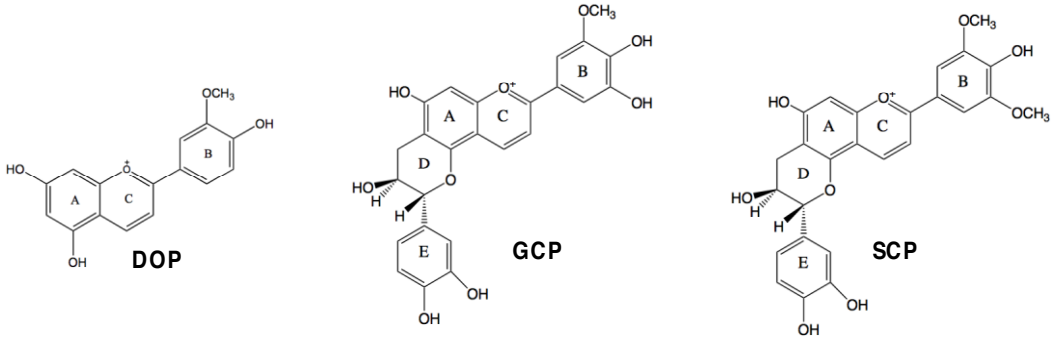

Fig. 29 - The model compound Deoxypeonidin (DOP) and the derivatives Guaiacylcathechinpyrylium (GCP), and Syringylcatechinpyrylium (SCP).

The formation and synthesis of the catechinpyrylium compounds SCP and GCP was previously described and these compounds named oaklins, a new class of brick-red colored pigments resulting from the reaction between catechin and wood aldehydes (de Freitas et al. 2004, Sousa et al. 2005). The formation of these pigments was also performed in a wine model solution and one of these (GCP) was also found in real wines aged in oak barrels, confirming that this type of compounds may contribute to the overall color changes observed during the aging process. Oppositely to anthocyanins, these pigments do not possess a glucose group in the flavylium core and are classified as deoxyanthocyanidins. To test and compare the properties of these compounds, a structurally simpler deoxyanthocyanidin (DOP) was also synthesized.

There is interest in knowing how the structural modifications carried out in the model compound DOP to give GCP and SCP affect the network of chemical reactions reported

above, and in particular the photochromic properties, as well as to make an argument regarding the stability of these compounds and their putative role in color changes in wine aging when compared to common wine anthocyanins. Furthermore, deoxyanthocyanidins are reported to be much more stable in slightly acidic solutions than anthocyanins and anthocyanidins, which points to the potential advantage of this type of compounds as viable commercial food colorants, and justifies the research developed in the chemistry of 3-deoxyanthocyanins and, in particular, the search for new colorants with significant stability (Iacobucci and Sweeny 1983, Dangles and Elhajji 1994, Sousa, Mateus 2012).

- MATERIALS AND METHODS

Synthesis of GCP (guaiacylcatechinpyrylium), SCP (syringylcatechinpyrylium) and deoxypeonidin. Regarding the synthesis of GCP, catechin ((2*R*,3*S*)-2-(3,4-dihydroxyphenyl)-3,4-dihydro-2*H*-chromene-3,5,7-triol) (8mM) was incubated with coniferaldehyde ((E)-3-(4-hydroxy-3-methoxyphenyl)prop-2-enal) (8mM) in 100 mL of a 12% ethanol-water solution (v/v) at pH 1.5 at 35ºC. The formation of the compound was followed over time by HPLC–DAD using a reversed phase C-18 (Merck) column (250 x 4.6 mm i.d., particle size 5 µm) at 25 ºC. Solvents were (A) water/formic acid (95:5) and (B) acetonitrile. The elution gradient was performed using L-2130 Merck pump from 10% to 35% B for 55 min at a flow rate of 1.5 mL min^{-1}. When the reaction was completed, after 10 days the sample was applied on a silica gel C-18 reversed phase SPE cartridge in order to remove inorganic salts and other impurities and the pigments were eluted with acidified methanol (2% v/v). Methanol was evaporated in a rotary evaporator at 38 °C, and the sample was freeze-dried and stored at −18 °C until use. The sample was further applied into a 5 cm diameter medium porosity sintered glass funnel with TSK Toyopearl gel HW-40(S) (Tosoh), connected to standard vacuum filtration glassware and gradually eluted with increasing percentages of acidified methanol (F1, 30%; F2, 40%; F3, 50%; F4, 60%; and F5, 80%). The criterion used for changing the percentages of methanol was the decrease in color intensity of the solution eluted from the column. Semipreparative HPLC was performed in order to further isolate and purify the GCP compound, eluted in fraction F2 from Toyopearl gel. This fraction was injected into a reversed phase C-18 (Merck) column (250 × 4.6 mm i.d., particle size 5 µm) at room temperature (volume injected was 1 mL). Solvents were (A) water/formic acid (95:5) and (B) acetonitrile. The elution gradient was performed using a L-2130 Elite LaChrom pump from 10 to 35% B for 65 min at a flow rate of 1.5 mL min^{-1} and detection was carried out

at 500 nm using a L-2420 Elite LaChrom detector. The synthesis of syringilcatechinpyrylium (SCP) was performed using the same procedure.

The synthesis of the compound 3-deoxypeonidin was followed according to procedure described in the literature (Sousa et al. 2012). Phloroglucinol (8mM) was incubated with coniferaldehyde (80mM) in a 100 mL 12% ethanol-water solution (v/v) at pH 1.0 at 35ºC. When the reaction was completed, after 4 days the sample was applied on a silica gel C-18 reversed phase SPE cartridge in order to remove inorganic salts and other impurities and the pigments were eluted with methanol acidulated with 2% HCl. The sample was further applied into a 300 x 16 mm i.d. TSK Toyopearl gel HW-40(S) (Tosoh, Japan) column and eluted with 40% aqueous methanol in 4 hours.

UV/Vis absorption spectra were recorded on a Varian Cary 100 Bio and Varian Cary 5000 spectrophotometers. The stopped flow experiments were conducted in an Applied Photophysics SX20 stopped-flow spectrometer provided with a PDA.1/UV photodiode array detector with a minimum scan time of 0.65 ms and a wavelength range of 200 nm to 700 nm. Flash photolysis was carried out as reported elsewhere (Pina, Melo 2001): to monitor the transient species a common spectrophotometer with a slightly modified compartment was used: (i) a slit (5 mm wide and 20 mm high) was opened on the external side of the sample holder in order to perform light excitation perpendicular to the analyzing beam; (ii) the whole sample compartment shielded (except for the slit described above) with black cardboard and black tape, to reduce as much as possible the flash light entering the exit slits. The time driven acquisition mode of the spectrophotometer was used and the traces were obtained each 5 nm or less according to the accuracy needed. From the traces, the time dependent absorption spectra were calculated.

- RESULTS AND DISCUSSION

Deoxypeonidin (DOP). Fig. 30 shows the spectral variations of the model compound deoxypeonidin, 5,7,4′-trihydroxy-3′-methoxyflavylium (DOP), occurring immediately after a pH jump from stock solutions at pH=1 to higher pH values (direct pH jumps). The spectral modifications are compatible with a triprotic acid with pK_a's equal to 3.9, 6.5 and 8.2, Table 3. The absorption spectra of the four species were determined by mathematical decomposition, Fig. 30D. The first deprotonation is likely to occur in position 7, a behavior early reported by Jurd and Geissman (Jurd and Geissman 1963) and confirmed in other anthocyanins (Pina et al. 2012). The second deprotonation most

probably occurs at position 4 and the third deprotonation at position 5. The pK_a of the first deprotonation compares with 4.1 for the analog luteolinidin (5,7,3′4′-tetrahydroxyflavylium) (Melo, Moura 2000), and 4.2 for apigeninidin (5,7,3′-trihydroxyflavylium) (Brouillard et al. 1982).

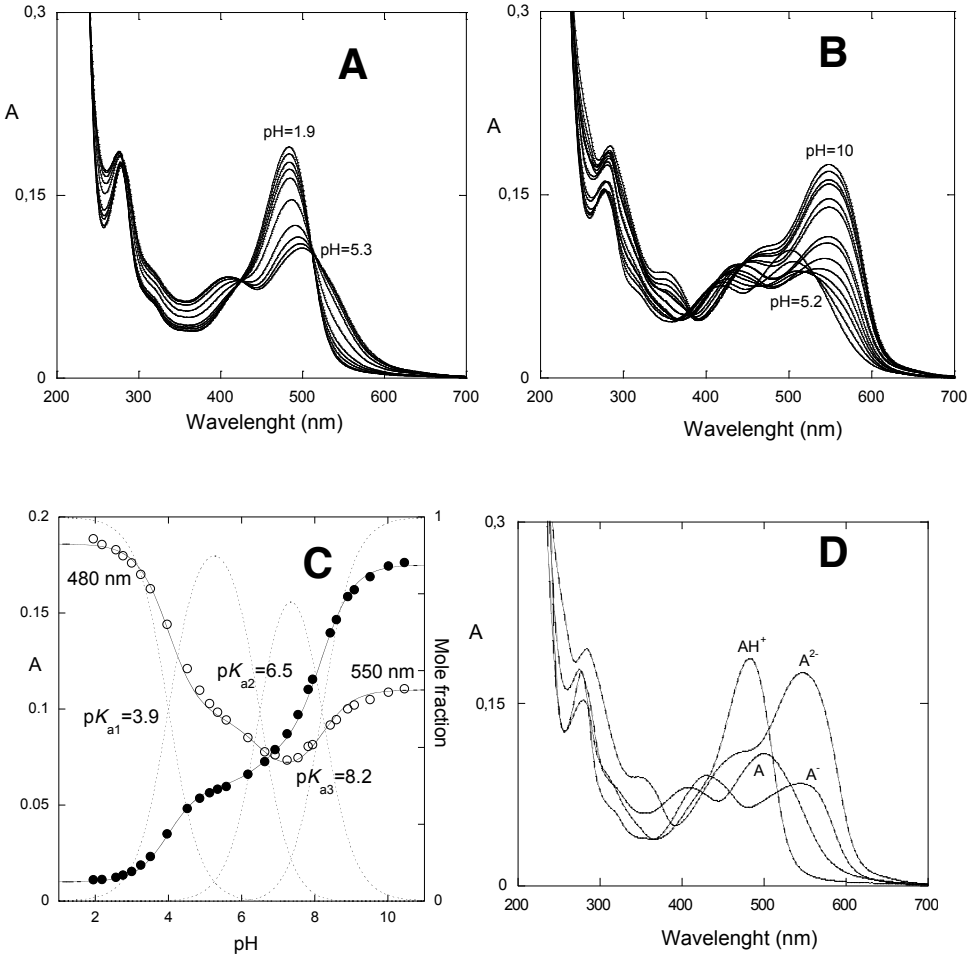

Fig. 30 - A - Spectral variations of the compound deoxypeonidin immediately after a pH jump from 1 to the range 1.9<pH<5.3; B - the same for 5.3<pH<10; C - Fitting of the absorption at 480 nm and 550 nm as a function of pH; D - Absorption spectra of the flavylium cation, quinoidal base and ionized quinoidal bases obtained by mathematical decomposition.

Table 3. Ionization constants of DOP, GCP and SCP.

Compound	pK_{a1}	pK_{a2}	pK_{a3}
DOP	4.0±0.1	6.5±0.1	8.2±0.1
GCP	3.6±0.1	7.7±0.1	-
SCP	3.8±0.1	n.d.	-

n.d. – not determined

The pH dependent absorption variations taking place in equilibrated solutions of DOP are represented in Fig. 31. The data are compatible with a single acid base equilibrium, eq.(1), permitting to define pK'_a=3.6, a value that compares with 3.8 (Melo et al. 2000) and 4.0 (Brouillard et al. 1982), for luteolinidin and apigeninidin. The shape and position of the equilibrium species at moderately acidic medium are compatible with the presence of quinoidal base in equilibrium with *trans*-chalcone. The fraction of the base at the higher pH plateau is given by the ratio K_a/K'_a=0.5 which is in good agreement with the data of Fig. 31.

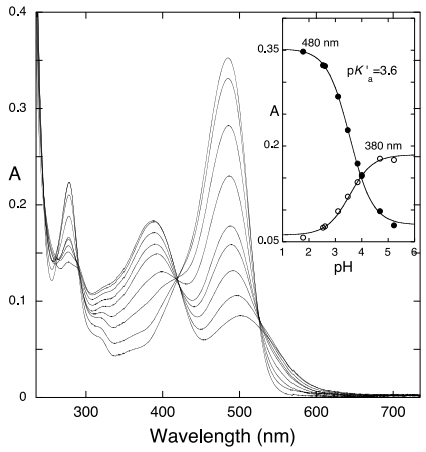

Fig. 31 - Spectra of equilibrated solutions of deoxypeonidin at different pH values, showing the equilibrium between AH^+, A and Ct; inset representation of the fitting obtained at two different wavelengths (pK'_a=3.6±0.1).

The relatively large mole fraction of the quinoidal base at the moderately acid equilibrium plateau when compared with anthocyanins (less than 5% in diluted solutions) (Pina et al. 2012, Petrov, Gavara 2013) or even 4′,7-dihydroxyflavylium (ca 10%) (Pina et al. 2012)

was found in the other deoxyanthocyanidins, such as luteolinidin and apigeninidin and in the related compound dragon's blood (4′,7-dihydroxy 5-methoxyflavylium) (Melo et al. 2007), where the quinoidal base is the major species and the *trans*-chalcone the minor one.

A series of pH jumps from pH=1.0 to higher pH values was carried out as shown in Fig. 32A for the final pH=4.7. The trace taken at 495 nm is compatible with a mole fraction of the base of 0.54 in good agreement with the previous calculations. Representation of the observed rate constants of these pH jumps as a function of pH leads to a bell shaped curve of Fig. 32B.

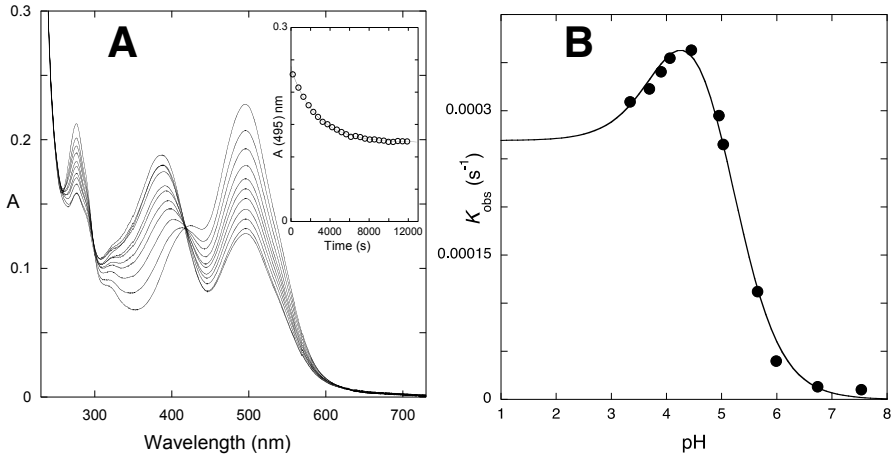

Fig. 32 - A - Spectral variations after a pH jump from 1 to 4.7 (k_{obs}=3.8x10^{-4} s^{-1}) - inset trace at 495 nm, the ratio between the final absorbance and initial one gives 0.54; B - representation of the observed rate constant as a function of pH. Fitting was achieved for pK_a=3.9; $K_h K_i k_i$=2.6×10^{-8}; k_{-i}=2.55×10^{-4} s^{-1}; $k_i K_i / k_{-h}$=6.65×10^{-6} M.

The bell shaped curve was previously reported for some flavylium derivatives and presupposes that the equilibrium between B and Cc is much faster than hydration and isomerization. For the present compound, it was not possible to observe the presence of B and Cc through a reverse pH jump (from solutions at higher pH to lower) monitored by stopped flow. As reported previously, the lack of a *cis-trans* isomerization barrier allows to apply the steady state approach for the kinetics, that converts Ct into AH^+/A and *vice versa*, eq.(6) and (7) (Pina et al. 1998, Petrov and Pina 2010, Pina, Petrov 2012).

$$\mathbf{Ct} \underset{\frac{K}{1+K_t}k_i}{\overset{k_{-i}}{\rightleftharpoons}} \mathbf{Cc} \rightleftharpoons \mathbf{B} \underset{\frac{[H^+]}{[H^+]+K_a}k_h}{\overset{\frac{k_{-h}}{1+K_t}}{\rightleftharpoons}} \mathbf{AH^+} \rightleftharpoons \mathbf{A} + \mathbf{H^+} \quad (6)$$

$$k_{obs} = \frac{\frac{[H^+]}{[H^+]+K_a} K_h K_t k_i + k_{-i}[H^+]}{\frac{K_t k_i}{k_h} + [H^+]} \quad (7)$$

The fitting of eq.(7) is represented in Fig. 32B and was achieved for pK_a=3.9; $K_h K_t k_i$=2.6×10^{-8}; k_{-i}=2.55×10^{-4} s^{-1}; $k_i K_t / k_{-h}$=6.65×10^{-6} M.

Differently from anthocyanins and anthocyanidins, deoxyanthocyanidins exhibit photochemistry and this is a powerful tool to contribute to the assessment of the rate and equilibrium constants of the system, in particular in compounds such as DOP where the reverse pH jumps are unable to detect the kinetic process involving B and Cc.

Fig. 33A shows the result of the steady state irradiation of DOP at 366 nm. The spectral variations show the appearance of AH^+/A at the expenses of the Ct disappearance. Flash photolysis, Fig. 33B, indicates that upon the flash two parallel processes occur: i) the forward reaction leading to the formation of flavylium cation, as indicated by the raising of the absorption at 493 nm (Fig. 33B up), ii) the backward reaction that partially recovers Ct, as shown by the recovery of the Ct absorption measured at 393 nm (Fig. 33B down). The rate constant measured in both traces is the same as expected for two parallel reactions. The data of the flash photolysis is easily comprehended if an energy level diagram is used (Fig. 34, see below how this diagram is obtained). After the flash, Ct disappears to give Cc (which absorbs much less at 393 nm) leading to a bleaching at this wavelength. Cc is not thermodynamically stable and has two ways to disappear, forward to give AH^+/A or backward to recover Ct.

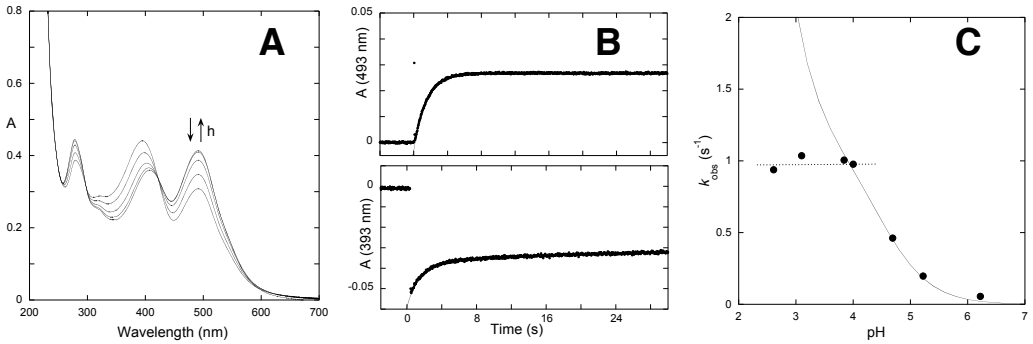

Fig. 33 - A - Spectral variations following the irradiation of DOP at pH=4.3 at the irradiation wavelength of 366 nm; B - flash photolysis of a solution of DOP, pH=4.4: flash photolysis showing the trace of the flavylium formation (493 nm) up and the recovery of the *trans*-chalcone (393 nm) down; C - representation of the rate constants of the flash photolysis process as a function of pH.

Representation of the rate constant as a function of pH, Fig. 33C, can be interpreted considering two regimes: at lower pH values the hydration is very fast and the tautomerization is the controlling step and the rate constants is given by eq.(8) (Mcclelland and Gedge 1980, Pina et al. 2001). At higher pH values the hydration is the rate determining step, and eq.(9) is verified (Gago, Petrov 2012). In eq.(8) the rate constant K_t^{OH} corresponds to the basic catalysis of the tautomerization constant (Brouillard et al. 1982), which in DOP is not present at these pH values, and by consequence at low pH values the rate constant is constant.

$$k_{obs(Taut)} = k_i + k_t + k_t^{OH}\ OH^- \tag{8}$$

$$k_{obs(Hyd)} = \frac{k_i K_t}{1+K_t} + \frac{[H^+]}{[H^+]+K_a} k_h + \frac{k_h [H^+]}{1+K_t} \tag{9}$$

A global fitting of the data including pK_a, pK'_a, eq.(7) to eq.(9) leads to the following set of equilibrium and rate constants, Table 4 and Table 5.

Table 4. Equilibrium constants.

Compound	pK'_a	K_h (M^{-1})[a)]	K_t [a)]	K_i [a)]
DOP	3.6±0.1	$1.7x10^{-7}$	1.7	350
GCP	3.05±0.1	$4.6x10^{-7}$	1.5	$1.3\ x10^{3}$
SCP	3.2±0.1	$1.0\ x10^{-7}$	4.3	$2.3\ x10^{3}$

a) Estimated error: 20%

Table 5. Rate constants.

Compound	$k_h(s^{-1})$	$k_{-h}(M^{-1}s^{-1})$	$k_t(s^{-1})$	$k_{-t}(s^{-1})$ [a)]	$k_i(s^{-1})$ [a)]	$k_{-i}(s^{-1})$
DOP	0.004	$2.3x10^{4}$	1.6	0.9	0.09	$2.55x10^{-4}$
GCP	0.013	$2.8x10^{4}$	1.8	1.2	0.36	$2.8x10^{-4}$
SCP	0.002	$2.0x10^{4}$	1.3	0.3	0.7	$3.1x10^{-4}$

a) Estimated error: 20%

Other set of solutions differing slightly from those reported in Table 4 and Table 5 are also possible, in particular in which regards the values of the tautomerization constants, due to the lack of its direct calculation from the reverse pH jumps.

The equilibrium constants calculated above permit the drawing of an energy level diagram (Fig. 34), which is very useful to the comprehension of the network of chemical reactions, as mentioned above.

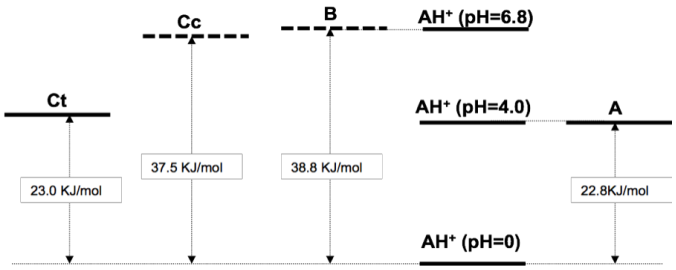

Fig. 34 - Energy level diagram for DOP based on the equilibrium constants of Table 3. The energy levels of B and Cc were obtained through the fitting of eqs.(7) to (9) due to the lack of observation of traces corresponding to B and Cc in the reverse pH jumps experiments carried out by stopped flow.

Guaiacylcathechinpyrylium (GCP). The absorption spectra obtained immediately after a pH jump from equilibrated solutions of GCP 1.6×10^{-4} M (10% EtOH) at pH=1.0 to the range 1.6<pH<5.2, Fig. 35A and 5.2<pH<9, Fig. 35B, are compatible with the formation of two quinoidal bases, likely to result from the deprotonation of the flavylium core in position 7 and 4′, Fig. 35C. The alternative deprotonation at the catechol substituent is not expected to affect significantly the absorption spectra, as observed in Fig. 35B, due to the lack of conjugation with the benzopyrilium ring. The absorption spectra of the species AH^+, A and A^-, were obtained by mathematical decomposition, Fig. 35D (Antonov and Petrov 2002).

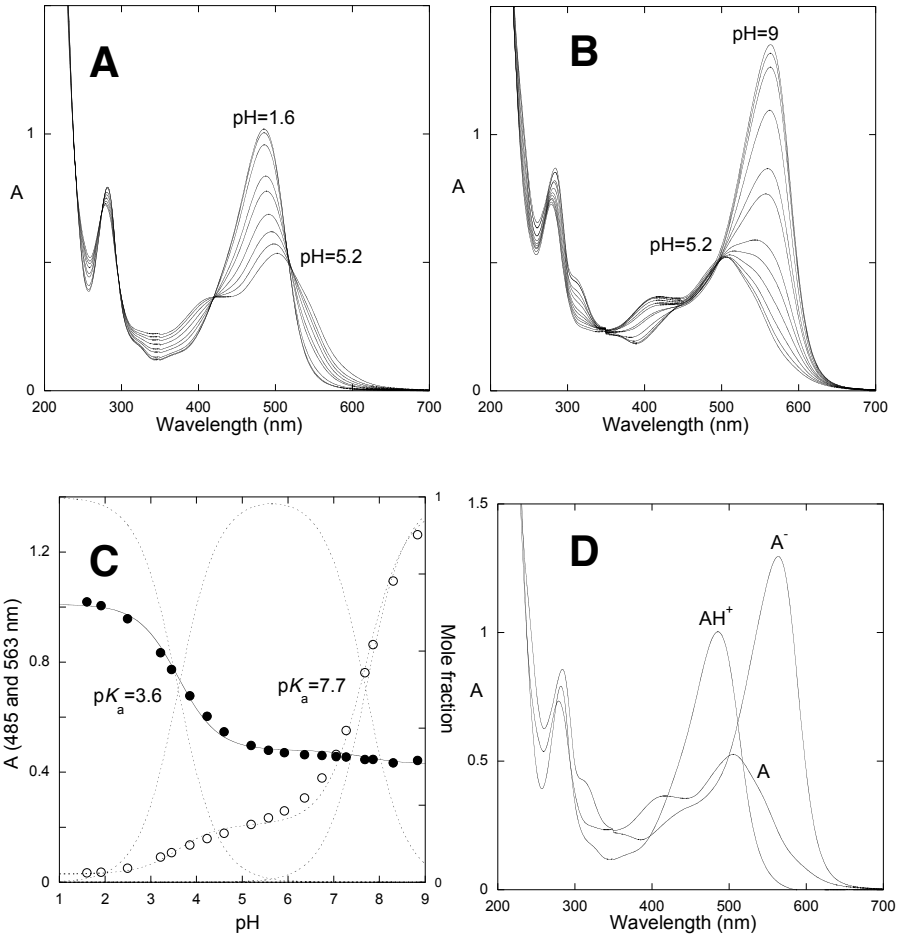

Fig. 35 - A - Spectral variations of the compound guaiacylcatechinpyrylium (GCP) 1.6×10^{-4} M (10% EtOH) immediately after a pH jump from 1 to the range 1.6<pH<5.2; B - the same for 5.2<pH<9; C - Fitting of the absorption at 485 nm and 563 nm as a function of pH; D - Absorption spectra of the flavylium cation, quinoidal base and ionized quinoidal base obtained by mathematical decomposition.

The solutions of GCP reported in Fig. 35 after equilibrium are shown in Fig. 36. The shape and position of the absorption spectra are compatible with a pH dependent equilibrium involving the acidic species flavylium cation and a mixture of quinoidal base and *trans*-chalcone at higher pH values, with pK'_a=3.05. This behavior is very similar to the parent compound DOP, where the equilibrium also involves essentially the same major species, **AH$^+$**, **A** and **Ct**.

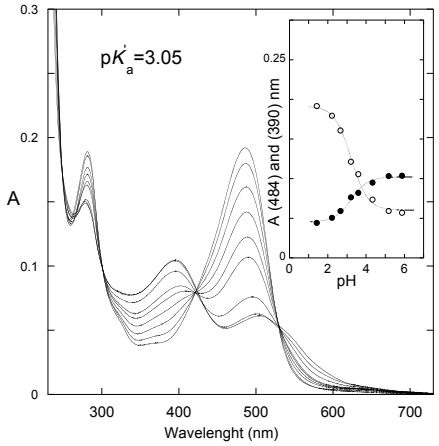

Fig. 36 - Spectra of equilibrated solutions of GCP (A) at different pH values, showing the equilibrium between AH^+, A and Ct; inset representation of the fitting obtained at two different wavelengths (pK'_a=3.05±0.1).

The kinetic processes from the initial state reported in Fig. 35 to the equilibrium, Fig. 36, are described in Fig. 37A. The spectral changes indicate that at pH=5.2 the quinoidal base initially formed through a pH jump partially disappears to give the *trans*-chalcone. The kinetics follows a mono-exponential decay. The rate constants measured at different pH values are reported in the inset of Fig. 37A, and follow the previously reported bell-shape curve. Fitting was achieved for the following parameters $K_hK_tk_i$=2.5x10^{-7} M^{-1}; k_{-i}=0.00028 s^{-1}; k_iK_t/k_{-h}=1.9x10^{-5} M. From the ratio $K_hK_tk_i$ /(k_iK_t/k_{-h}), k_h=0.013 s^{-1}.

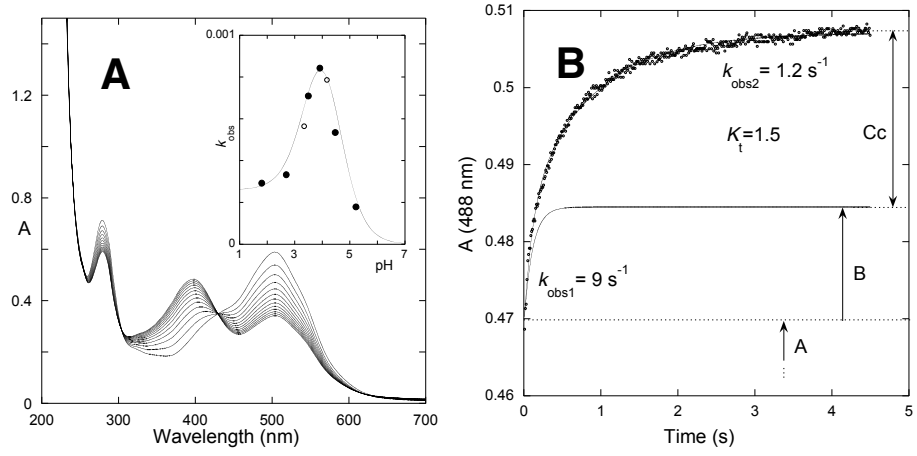

Fig. 37 - A - Spectral variations of GCP after a pH jump from 1 to 5.2 (k_{obs}=1.8x10^{-4} s-1): inset-representation of the observed rate constant as a function of pH. Fitting was achieved for pK_a=3.6; $K_hK_tk_i$=2.5x10^{-7} M^{-1}; k_{-i}=0.00028 s^{-1}; k_iK_t/k_{-h}=1.9x10^{-5} M. From the ratio $K_hK_tk_i$ /(k_iK_t/k_{-h}), k_h=0.013 s^{-1}; (●) direct pH jumps (○) thermal recovery of the photoproduct, see below Fig. 38; B - Reverse pH jump from solutions aged about 5 minutes at pH=5.4 and back to pH=1.2 (K_t=1.5).

A first pH jump on stocked solutions at pH=5.14 was carried out, the solutions kept during 5 min at this pH value and a reverse pH jump to 1.2 was performed allowing the monitoring of the flavylium appearance by stopped flow, Fig. 37B. The traces indicate that all the quinoidal base present at pH=5.4 was converted during the mixing time of the stopped flow, and the flavylium grows according to a bi-exponential kinetics. The fast process corresponds to the hydration reaction, which at pH=1.2 is faster than tautomerization due to its proton dependence. The slowest step corresponds to the formation of more flavylium cation from Cc through B and it is assigned to the rate constant k_{-t}=1.2 (Pina et al. 2012). Moreover, from the ratio of amplitudes of both kinetics the constant K_t=1.5 was calculated, allowing to obtain k_t=1.8 and K_h K_i=8.6x10^{-4}.

Similarly to DOP the compound GCP is photochromic, Fig. 38. Irradiation of the *trans*-chalcone leads to the formation of quinoidal base with a quantum yield of 0.1. The system is reversible and thus a photostationary state is achieved. The kinetics of the thermal recovery of the photoproduct was measured at two pH values and fits quite well with the bell shape curve, Fig. 37A. Flash photolysis of equilibrated solutions at pH=4.1 is similar to the parent compound. Within the lifetime of the flash Ct disappears to give Cc, which goes forward to form AH^+/A or backward to recover Ct. Representation of the observed rate constants from experiments like the one of Fig. 38B at different pH values are shown in Fig. 38C. A global fitting was achieved through eq. (8) and eq.(9) and the one of the bell-shaped curve, eq.(9) leading to the rate and equilibrium constants presented in Table 4 and Table 5.

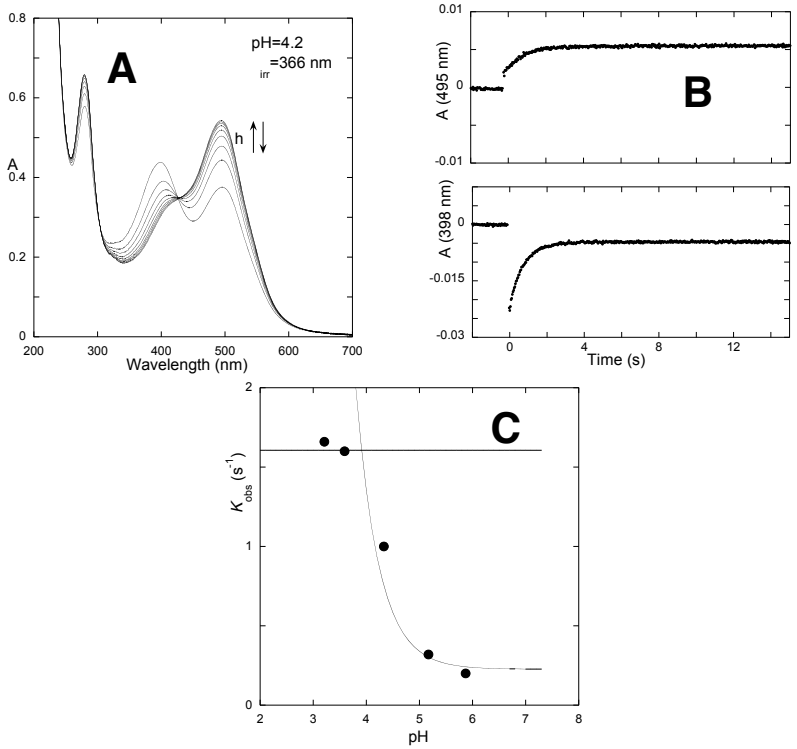

Fig. 38 - A - Spectral variations following the irradiation of GCP at pH=4.1 at the irradiation wavelength of 366 nm; B - Flash photolysis followed at 495 nm (AH^+ and A) up and 398 nm (Ct) bottom; C - observed rate constant of the flash photolysis as a function of pH.

As in the case of DOP it is easy to construct an energy level diagram where the five species are relatively positioned, Fig. 39. It is worth of note the fact that similarly to DOP (and to SCP see below) the energy level of Ct and A are close and lower relatively to B and Cc, as expected from the shape and position of the absorption spectra at the equilibrium.

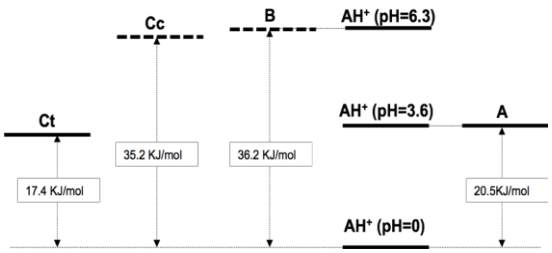

Fig. 39 - Energy level diagram for GCP based on the equilibrium constants of Table 3.

Syringylcatechinpyrylium (SCP). The absorption spectra of SCP was obtained immediately after the pH jump and a value of pK_{a1}=3.8 was obtained in Fig. 40.

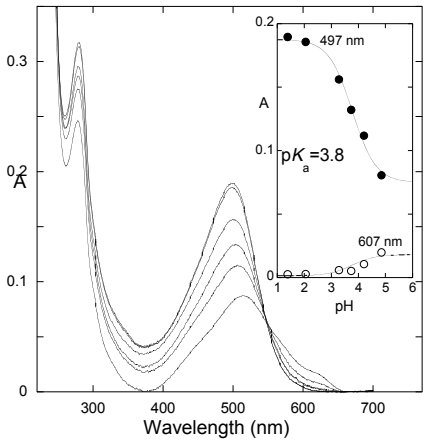

Fig. 40 - Absorption spectra of solutions of SCP taken immediately after a direct pH jump from stock solutions at pH=1 to higher pH values; inset representation of the fitting obtained at two different wavelengths (pK_a=3.9±0.1).

The further ionization constants were not determined due to the appearance of some decomposition, which can be attributed to the presence of the cathecol unit, which is known to be easily oxidized at higher pH values. The absorption spectra of the equilibrated solutions, Fig. 41, are again compatible with an equilibrium involving essentially the species AH^+, A and Ct.

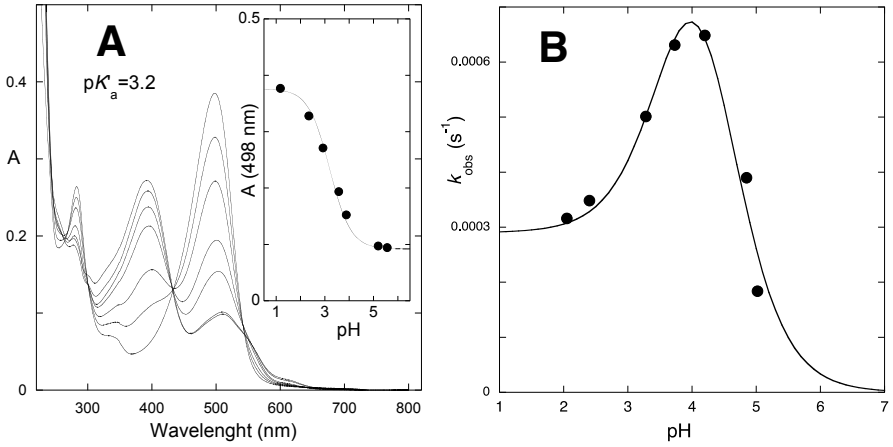

Fig. 41 - A - Absorption spectra of equilibrated solutions of SCP; B - Bell shaped curve for the compound SCP.

The spectral variations upon irradiation of SCP of equilibrated solutions at pH=4.4 are shown in Fig. 42, and it can be concluded that SCP shows a behavior similar to DOP and GCP. However there are some differences, which can be easily visualized from the data of Fig. 43C, Fig. 38C and Fig. 42C. Inspection of eq.(9) used to fit the data indicate that it tends to eq.(10) when the proton concentration decreases.

$$k_{obs(hydr)} = \frac{k_i K_t}{1 + K_t} \qquad (10)$$

The value of eq.(10) defines the plateau reached in the above mentioned figures, respectively 0.057, 0.22 and 0.63. This is also in accordance with the data reported in Table 3 and Table 4.

Flash photolysis of equilibrated solutions at pH=4.4 is similar to the parent compound and GCP. Within the lifetime of the flash Ct disappears to give Cc, which goes forward to form AH^+/A or backward to recover Ct. Representation of the observed rate constants from experiments like the one of Fig. 42B at different pH values are shown in Fig. 42C.

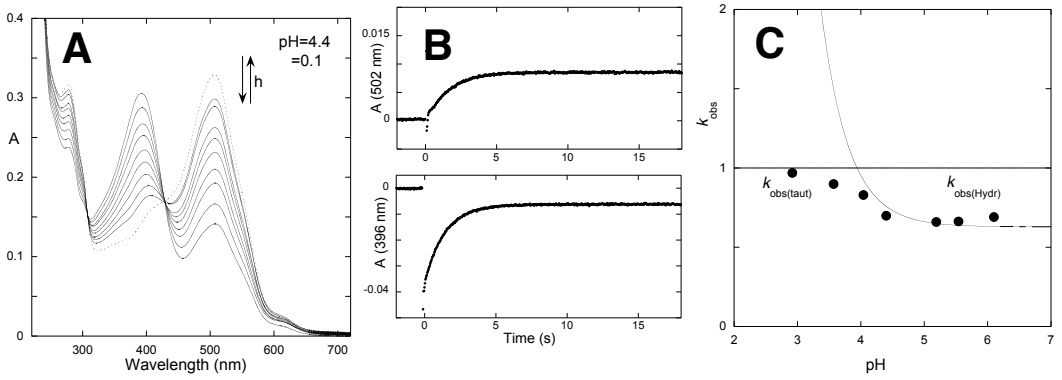

Fig. 42 - A - Irradiation of the compound SCP at pH=1.0 (full lines): the spectrum of the solution immediately after its preparation from a pH jump from pH=1.0 to pH=4.4, before being irradiated is also shown (traced line); B - flash photolysis traces at 502 nm (AH^+/A) and 396 nm (Ct); C - observed rate constant of the flash photolysis as a function of pH.

The energy level diagram of SCP is shown in Fig. 43 and is qualitatively similar to DOP and GCP showing the higher stability of the species A and Ct at higher pH values, much higher than B and Cc.

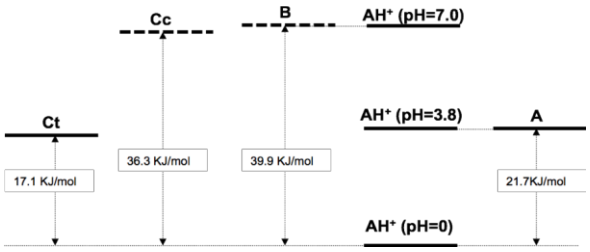

Fig. 43 - Energy level diagram for SCP based on the equilibrium constants of Table 3. The energy levels of B and Cc were obtained through the fitting of eqs.(7) to (9) due to the lack of observation of traces corresponding to B and Cc in the reverse pH jumps experiments carried out by stopped flow.

- CONCLUSION

The network of chemical reactions involving anthocyanins and related compounds is pH dependent and by consequence the detailed kinetics of the system have been obtained through pH jumps, which allow us to follow the relaxation processes toward the new equilibrium. When the flavylium derived networks of chemical reactions exhibit photochemistry flash photolysis is an excellent complementary technique to collect kinetic information on the system without changing the pH. This is particularly important for flavylium derivatives possessing a low *cis-trans* isomerization barrier, since hemiketal, B, and *cis*-chalcone, Cc, are transient species not detected from the pH jump studies. In fact, when compared to common wine anthocyanins in which B (incolor) is the major species at higher pH values, sometimes at the pH of the wine, these oaklin compounds have a behavior more similar to simpler deoxyanthocyanidins, in which the equilibrium involves essentially AH^+, A and Ct and the mole fraction of the quinoidal base A is approximately 50%. Furthermore the pK′a values obtained for GCP and SCP (3.05 and 3.2, respectively) are higher than the ones reported for malvidin-3-glucoside (2.54 (Brouillard et al. 1978) and 2.3 (Nave, Petrov 2010)), meaning that the flavylium cation is more stable in the former. The lost of color in solution is thereby greatly diminished in oaklin compounds when compared to other anthocyanins, not only because pK'_a is greater but also because the mole fraction of the base is substantially higher at higher pH values. In consequence, oaklins which are formed through the reaction of catechin and oak wood aldehydes, may play an important role in some color changes observed in wine aging and may contribute to the overall color presented by some wines. Nevertheless, it is important to note that anthocyanins like malvidin-3-glucoside are still present in wines at concentration levels far higher than oaklins and their contribution to

wine color must not be underestimated, neither the effects of copigmentation (Boulton 2001).

Despite some differences, the general behavior of guaiacylcathechinpyrylium and syringylcatechinpyrylium is quite similar to the model compound deoxypeonidin. Given the fact that the lost of color in solution of these compounds is lower than anthocyanins they may be regarded as potential commercial food colorants. However, further studies should be performed in order to test their stability to other conditions (temperature, sulfites, etc) as well as to study their antioxidant properties.

- AKNOWLEDGEMENTS

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Chapter 4

Deoxyvitisins: a new set of pyrano-3-deoxyanthocyanidins

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In this work, the first author was responsible for undertaking all experimental activities with the assistance of Paula Araújo in the purification of the new compounds. He also benefited from the advice of MS and NMR technicians and guidance by the rest of the authors.

Deoxyvitisins: a new set of pyrano-3-deoxyanthocyanidins

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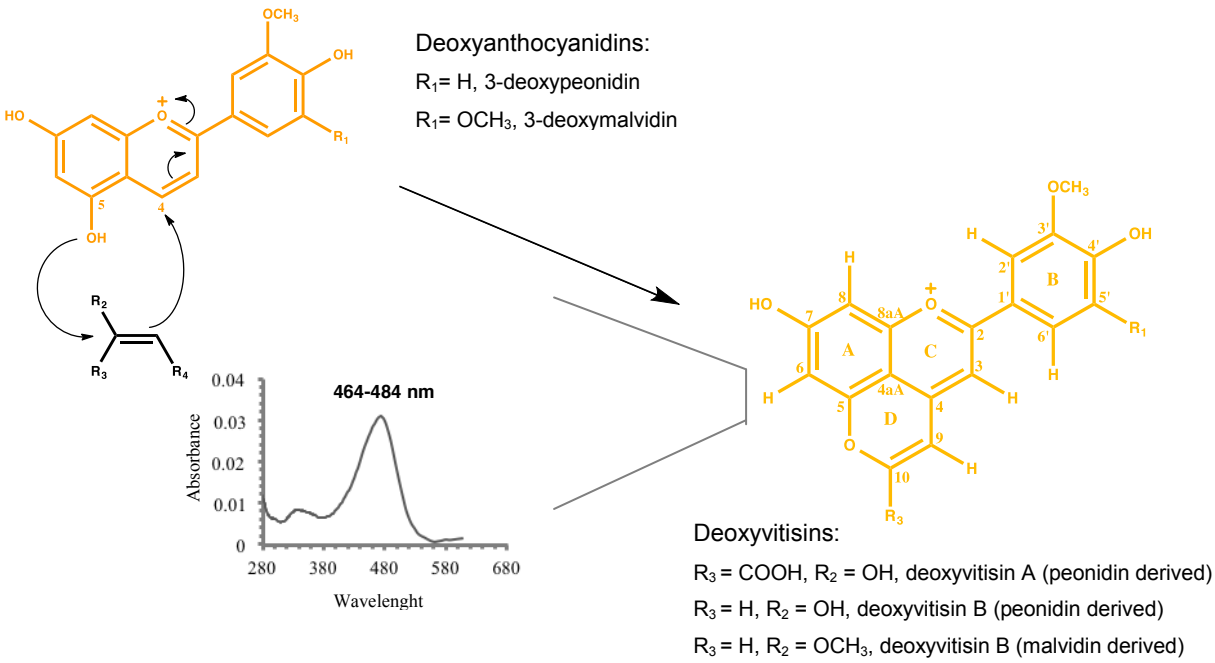
The chemical synthesis of deoxyvitisins (pyrano-3-deoxyanthocyanidins) is reported herein for the first time. Three different types of compounds were synthesized from the reaction between two deoxyanthocyanidins (deoxypeonidin and deoxymalvidin) and pyruvic acid, vinyloxy-trimethylsilane and acetone-1,3-dicarboxylic acid. The structure of the new compounds has been characterized by means of visible, MS and NMR spectroscopy.



KEYWORDS: deoxyvitisins; vitisins; deoxyanthocyanidins; anthocyanidins; aldehydes; vinyloxy-trimethylsilane; pyruvic acid; acetone-1,3-dicarboxylic acid; NMR; mass spectrometry

- INTRODUCTION

Pyranoanthocyanins belong to an important group of anthocyanin-derived pigments that occur essentially in fruits and processed foodstuffs like vegetable juices or red wines (Brouillard, Chassaing 2003, Fulcrand, Atanasova 2004, Rentzsch, Schwarz 2007). Formation of this kind of compounds in food matrixes result from a cycloaddition between C-4/5-OH of the anthocyanin and a double bond from another molecule such as acetaldehyde (Bakker and Timberlake 1997), pyruvic acid (Fulcrand et al. 1998),

vinylcatechin (Cruz, Teixeira 2008) and vinylcatechol (Hakansson, Pardon 2003, Schwarz, Jerz 2003), resulting in an additional pyran ring. Vitisins of type A differ from vitisins B by the presence of a carboxylic group in the additional pyranic ring (Bakker and Timberlake 1997, Mateus, Silva 2001, Morata, Calderon 2007, Cruz et al. 2008, Oliveira, de Freitas 2009). Due to their new pyranic ring, they are much more stable towards pH variations and bleaching by SO_2 in comparison to the genuine anthocyanins (Mateus et al. 2003).

Oppositely to anthocyanins, deoxyanthocyanidins are yellowish pigments commonly found in several food plants such as corn, black tea leaves, and sorghum and they do not possess a glycose group. They also have a significant increased stability in slightly acidic solutions compared to anthocyanins and several studies have demonstrated the potential applications of these compounds for viable commercial food colorants, hair dyes, laser dyes, sensitizers for solar cells, molecular-level memory systems and health-promoting phytochemicals (Czerney et al. 1995, Cherepy et al. 1997, Roque et al. 2002, Awika, Rooney 2005, Shih, Siu 2007).

Previously, successful attempts have been made to synthesise pyranoflavylium derivatives by the condensation of a 5-hydroxy-4-methylflavylium cation and an adequate aldehydic partner, which in some cases result in vitisidin type structures (aglycones of vitisin) (Roehri-Stoeckel, Gonzalez 2001, Chassaing, Isorez 2008). Recently, a new pigment was isolated from red *Sorghum bicolor* with a structure of a pyrano-3-deoxyanthocyanidin, containing apigeninidin as a base unit (Khalil et al. 2010). This was the first report of a deoxyvitisin-type compound, which displayed a higher stability comparetively to the corresponding anthocyanin, justifying the research developed in the chemistry of 3-deoxyanthocyanins and, in particular, the search for new colorants with significant stability.

Bearing this, the aim of this work was to synthesize deoxyvitisins of type A, B and methylpyranodeoxyanthocyanidins and to propose a formation mechanism.

- MATERIALS AND METHODS

The synthesis of deoxyvitisins derived from deoxypeonidin was performed with the reagents pyruvic acid, vinyloxy-trimethylsilane and acetone-1,3-dicarboxylic acid in separate model aqueous solutions, as described elsewhere (He, Santos-Buelga 2006, Oliveira et al. 2009), whereas the synthesis of one deoxyvitisin derived from deoxymalvidin was tested with the reagent viniloxy-trimethylsilane. The synthesis of the reagents deoxypeonidin and deoxymalvidin is described elsewhere (Sousa et al. 2012). The formation of new compounds was followed by HPLC-DAD using a reversed phase

C-18 (Merck®) column (250 x 4.6 mm i. d., particle size 5 µm), at 25 ºC. Solvents were (A) water/formic acid (95:5) and (B) acetonitrile. The elution gradient was performed using a L-2130 Merck® pump from 10 to 35% B for 55 min at a flow rate of 1.5 $mL.min^{-1}$. The yield obtained for the reactions was around 30% and the maximum concentration of the compounds was achieved between 3 and 5 days of reaction.

- RESULTS AND DISCUSSION

The mass spectra of these compounds obtained by LC–DAD/ESI/MS in the positive ion mode showed molecular ions compatible with the structures of deoxyvitisins of type A, B and methylpyranodeoxypeonidin from the reactions with pyruvic acid, vinyloxy-trimethylsilane and acetone-1,3-dicarboxylic acid, respectively (Table 6, Fig. 44).

Table 6. Molecular ions and respective MS^2 (of the molecular ion) and MS^3 (of the main fragment in MS^2) fragmentations from deoxyvitisin A, B and methylpyranodeoxypeonidin. λ_{max} obtained from the visible spectrum of deoxyvitisin A, deoxyvitisin B and methylpyranodeoxypeonidin.

	Deoxyvit A	**Deoxyvit B**	**Methylpyrano**
MS ($[M]^+$)	353 m/z	309 m/z	323 m/z
MS^2 ($[M-15]^+$)	338 m/z	294 m/z	308 m/z
MS^3 ($[M-15-28]^+$)	310 m/z	266 m/z	280 m/z
λ_{max}	464 nm	474 nm	484 nm

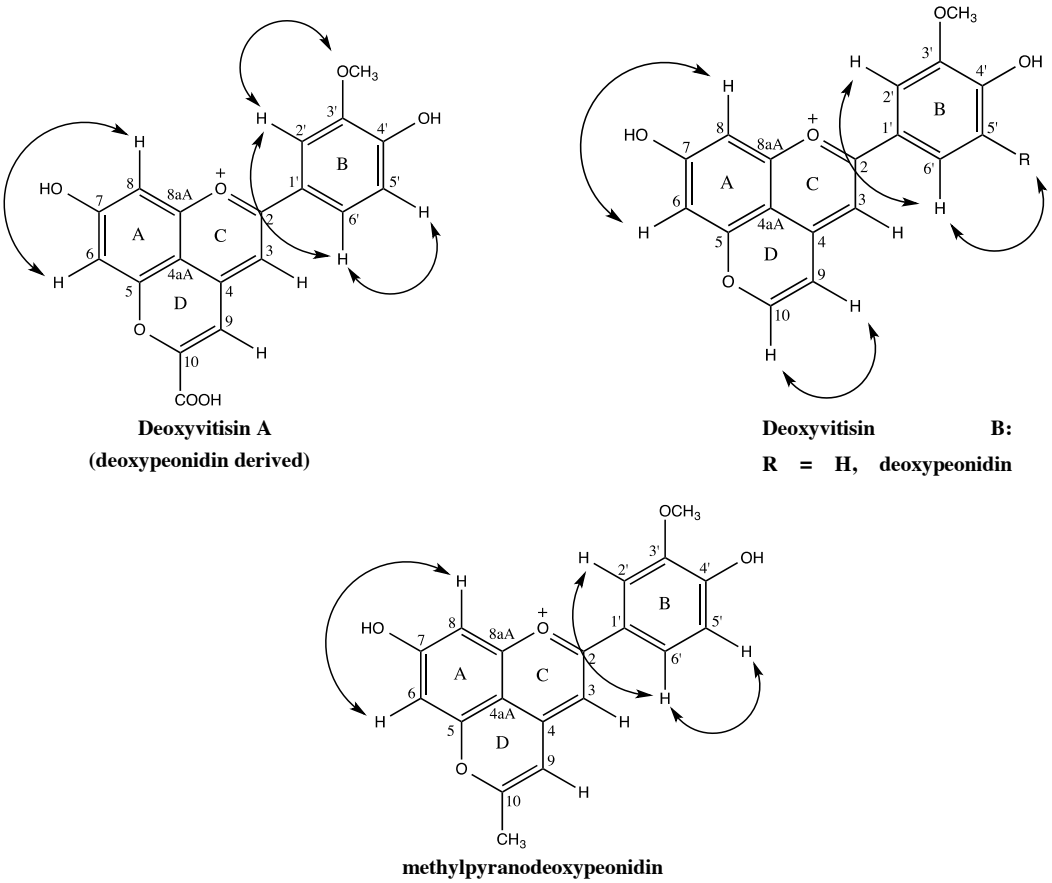


Fig. 44 - Chemical structure of the newly sinthesised deoxyvitisins A, B and methylpyranodeoxypeonidin (represented with COSY spectrum correlations).

In addition, the MS^2 spectra for all the compounds showed a fragment that correspond to the loss of a methyl group, $[M-15]^+$ and further MS^3 fragmentation of this ion yielded a fragment at m/z $[M-15-28]^+$, corresponding to the loss of CO. The fragmentation pattern observed for these compounds was similar to the one described in the literature for the reagent deoxypeonidin (Sousa et al. 2012). The deoxyvitisin B derived from deoxymalvidin also showed a similar fragmentation pattern.

The reaction between deoxyanthocyanidins and the reagents studied led to the formation of new deoxyvitisins which showed a maximum absorption in the visible range between λ_{max} 464-484 nm with an hypsochromic shift towards their deoxyanthocyanidin precursors (487 nm for deoxypeonidin and 492 nm for deoxymalvidin (Sousa et al. 2012)), conferring them a yellow/orange color (Table 6).

The deoxyvitisins derived from deoxypeonidin were synthesised and their structure was fully elucidated by 1H and 13C NMR spectroscopy (Table 7) using 1D and 2D techniques (COSY, HSQC, HMBC) (available in Supplementary Data).

For all the compounds, protons 6A and 8A revealed a clear correlation in the COSY spectrum (represented in Fig. 25) and were attributed to the doublets at δ 7.20/7.13/7.09 (deoxyvitisin A/B/methylpyrano) and 7.38/7.32/7.27 ppm (deoxyvitisin A/B/methylpyrano), respectively. Carbons 6A and 8A were assigned through HSQC correlations with the respective protons. Carbons 5A and 7A were attributed through their long-range $^{1}H - ^{13}C$ correlations with protons 6A (and 10D in the case of deoyvitisin B) and 8A, 6A, respectively, observed in the HMBC spectrum.

Carbon 4aA was assigned at δ 109.1/107.6 ppm (deoxyvitisin A and B/methylpyrano) from its long-range $^{1}H - ^{13}C$ correlations with protons 8A, 6A, 9D and 3C and the carbon 8aA at δ 154.0/153.5 ppm (deoxyvitisin A and B/methylpyrano) from its correlation with H-8A, observed in the HMBC spectrum.

The vinylic proton 3C was attributed to the singlet at δ 7.87/8.06/7.78 ppm (deoxyvitisin A/B/methylpyrano). The respective carbon was assigned through HSQC correlation with its proton and through a long-range $^{1}H - ^{13}C$ correlation with the proton H-9D in the HMBC spectrum. Carbon 2C was assigned at circa δ 169 ppm from its long-range 1H – ^{13}C correlations with protons H-3C, H-2'B and H-6'B observed in the HMBC spectrum. Carbon 4C was attributed to δ 150.9 ppm in the case of deoxyvitisin B through its correlation with proton H-10D and to δ 154.1 ppm in the case of methylpyranodeoxypeonidin, seen by proton H-3C.

Table 7. ^{1}H and ^{13}C NMR data of deoxyvitisin A, B and methylpyranodeoxypeonidin, determined in DMSO/TFA (90:10).

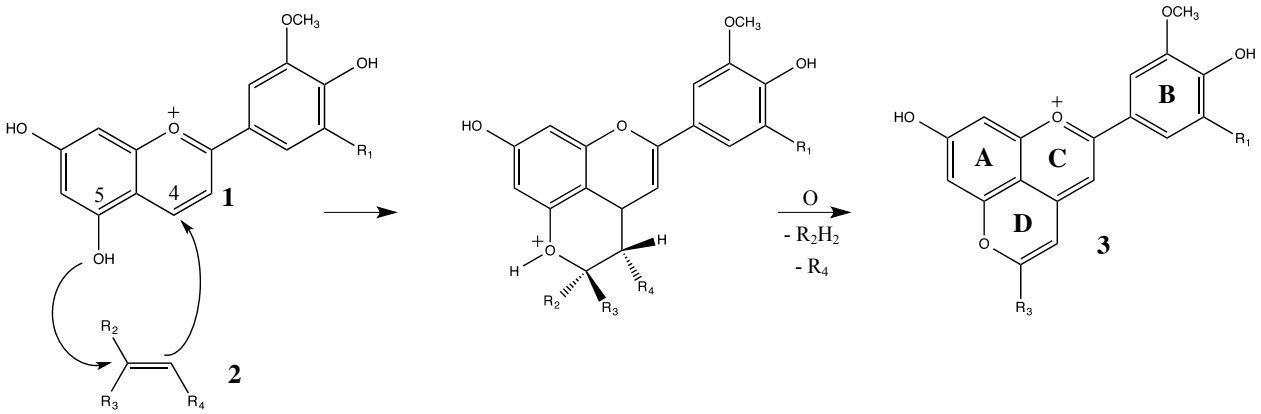
	Deoxyvitisin A (deoxypeonidin derived)		**Deoxyvitisin B (deoxypeonidin derived)**		**Methylpyranodeoxypeonidin**	
Position	**δ ^{1}H (ppm); *J* (Hz)**	**δ ^{13}C (ppm)**	**δ ^{1}H (ppm); *J* (Hz)**	**δ ^{13}C (ppm)**	**δ ^{1}H (ppm); *J* (Hz)**	**δ ^{13}C (ppm)**
Ring A, B, C						
2C		169.7		169.1		168.3
3C	8.06; s	103.3	7.87; s	101.8	7.78; s	101.1
4C		154.7		150.9		154.1
4aA		109.1		109.1		107.6
5A		153.0		153.4		153.8
6A	7.09; d (1.60)	101.1	7.13; d (1.38)	100.7	7.13; d (1.50)	100.4
7A		168.4		167.5		167.3
8A	7.27; d (1.60)	101.2	7.32; d (1.38)	100.9	7.32; d (1.50)	100.7
8aA		154.0		154.0		153.5
1′B		120.5		120.5		120.6
2′B	7.76; d (1.72)	111.7	7.76; d (1.72)	111.5	7.72; d (1.90)	111.5
3′B		149.1		148.9		148.8
4′B		155.2		154.6		154.2
5′B	7.08; d (8.57)	117.0	7.06; d (8.57)	116.8	7.06; d (8.43)	116.7
6′B	7.87; dd (1.72; 8.57)	124.4	7.85; dd (1.72; 8.57)	123.9	7.81; dd (1.90; 8.43)	123.5
OCH_3	3.94; s	56.3	3.93; s	56.2	3.91; s	56.3
Ring D						
9D	7.64; s	109.4	7.03; *	107.3	6.91; s	105.1
10D		154.5	8.47; d (5.14)	160.0		172.2
COOH		160.1	-	-	-	-
CH_3	-	-	-	-	2.57; s	21.2

s - singlet; d - doublet; dd – double doublet; * - unresolved

Concerning the new pyranic ring D of deoxyvitisin A, the proton H-9D was assigned to a singlet at δ 7.64 ppm. The carbon 10D and the carbon from the carboxylic group were attributed to δ 154.5 and 160.1 ppm, respectively, from their long distance correlations observed in the HMBC spectrum with proton H-9D. In deoxyvitisin B, the protons H-9D and H-10D revealed a clear correlation in the COSY spectrum. Proton H-10D was assigned to a doublet at δ 8.47 ppm and proton H-9D to an unresolved signal at δ 7.03

ppm. Carbon 10D was assigned at δ 160.0 ppm through HSQC correlation with its proton and a long-range $^{1}H - ^{13}C$ correlation with proton H-9D and carbon 9D at δ 107.2 ppm through HSQC and the long-range $^{1}H - ^{13}C$ correlations with protons H-3C and H-10D in the HMBC spectrum. In methylpyranodeoxypeonidin, the proton H-9D was assigned to a singlet at δ 6.91 ppm and its carbon at δ 105.1 ppm through HSQC correlation and long-range correlations with protons H-3C and CH_3. The protons from CH_3 were attributed to a singlet at δ 2.57 ppm. Carbon from CH_3 and 10D were assigned to δ 21.2 and 172.2 ppm, respectively, from their long distance correlations observed in the HMBC spectrum with protons H-9D and CH_3 in the case of carbon 10D.
These correlations identify unambiguously the position of the pyranic ring D linked to the pyrylium ring C and the aromatic ring A by carbons 4C and 5A.
The chemical shift of the remaining protons and carbons were easily established by HSQC and HMBC techniques. Likewise, the deoxyvitisin B derived from deoxymalvidin was also characterised (available in Supplementary Data).

The hypothetic mechanism of formation of these new pigments is represented in Fig. 45. By analogy with the mechanism described in the literature for the formation of pyranoanthocyanins (Oliveira et al. 2009), deoxyvitisins **3** should result from the cyclic addition of a compound in its enolic form **2** onto carbon 4 and an hydroxyl group at the carbon 5 of the deoxyanthocyanidin **1** (Fulcrand, Duenas 2006, Cruz et al. 2008), yielding a new pyranic ring D **3**, which is thought to be responsible for the higher stability in pyranoanthocyanins when compared to the original anthocyanins (Cruz et al. 2008). This farther extended conjugation of the π electrons is at the origin of their maximum absorption in the range between λ_{max} 464-484 nm with a slight hypsochromic shift comparatively to their deoxyanthocyanidin precursors.



R_1 = H: deoxypeonidin; R_1 = OCH_3: deoxymalvidin

R_2 = OH, R_3 = COOH, R_4 = H: pyruvic acid (enolic form)

R_2 = $OSi(CH_3)_3$, R_3 = H, R_4 = H: vinyloxy-trimethylsilane

R_2 = OH, R_3 = CH_2COOH, R_4 = COOH: acetone-1,3-dicarboxylic acid (enolic form)

Fig. 45 - Hypothetical mechanism for the formation of deoxyvitisins **3** obtained from the reaction between deoxyanthocyanidins **1** and carbonyl compounds **2** (enolic forms).

- CONCLUSION

These results show for the first time a new class of deoxyanthocyanidins that have interesting visible spectroscopic properties, representing a step forward towards the research for new pigments with significant stability. Nonetheless, further studies are still required to test their chromatic properties at different conditions (pH, temperature, sulfites, etc) as well as their antioxidant capability in order to precisely evaluate the usefulness of these compounds in several potential applications in the food, medical, cosmetic and/or technology industries.

- AKNOWLEDGEMENTS

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- SUPPLEMENTARY DATA

Table. 8. ^{1}H and ^{13}C NMR data and HMBC and HSQC correlations of deoxyvitisin A (peonidin derived), determined in DMSO/TFA (90:10).

Position	**δ ^{1}H (ppm); *J* (Hz)**	**δ ^{13}C (ppm)**	**HMBC**	**HSQC**
Ring A, B, C				
2C		169.7	H-3C, H-2′B, H-6′	
3C	8.06; s	103.3	H-9D	H-3C
4C		154.7	H-3C	
4aA		109.1	H-8A, H-6A, H-9D, H-3C	
5A		153.0	H-6A	
6A	7.09; d (1.60)	101.1	H-8A	H-6A
7A		168.4	H-6A, H-8A	
8A	7.27; d (1.60)	101.2	H-6A	H-8A
8aA		154.0	H-8A	
1′B		120.5	H-3C, H-5′B	
2′B	7.76; d (1.72)	111.7	H-6′B	H-2′B
3′B		149.1	OCH_3, H-2′B, H-5′B	
4′B		155.2	H-2′B, H-5′B, H-6′B	
5′B	7.08; d (8.57)	117.0		H-5′B
6′B	7.87; dd (1.72; 8.57)	124.4	H-2′B, OCH_3	H-6′B
OCH_3	3.94; s	56.3		OCH_3
Ring D				
9D	7.64; s	109.4		H-9D
10D		154.5	H-9D	
COOH		160.1	H-9D	

s - singlet; d - doublet; dd – double doublet

Table 9. ^{1}H and ^{13}C NMR data and HMBC and HSQC correlations of deoxyvitisin B (peonidin derived), determined in DMSO/TFA (90:10).

Position	**δ ^{1}H (ppm); *J* (Hz)**	**δ ^{13}C (ppm)**	**HMBC**	**HSQC**
Ring A, B, C				
2C		169.1	H-3C, H-2′B, H-6′B	
3C	7.87; s	101.8	H-9D	H-3C
4C		150.9	H-10D	
4aA		109.1	H-8A, H-6A, H-9D, H-3C	
5A		153.4	H-6A, H-10D	
6A	7.13; d (1.38)	100.7	H-8A	H-6A
7A		167.5	H-6A, H-8A	
8A	7.32; d (1.38)	100.9	H-6A	H-8A
8aA		154.0	H-8A	
1′B		120.5	H-3C, H-5′B	
2′B	7.76; d (1.72)	111.5		H-2′B
3′B		148.9	OCH_3, H-2′B, H-5′B	
4′B		154.6	H-2′B, H-5′B	
5′B	7.06; d (8.57)	116.8		H-5′B
6′B	7.85; dd (1.72; 8.57)	123.9	H-2′B	H-6′B
OCH_3	3.93; s	56.2		OCH_3
Ring D				
9D	7.03; *	107.3	H-3C, H-10D	H-9D
10D	8.47; d (5.14)	160.0	H-9D	H-10D

s - singlet; d - doublet; dd – double doublet, * - unresolved

Table 10. ^{1}H and ^{13}C NMR data and HMBC and HSQC correlations of deoxyvitisin B (malvidin derived), determined in DMSO/TFA (90:10).

Position	**δ ^{1}H (ppm); *J* (Hz)**	**δ ^{13}C (ppm)**	**HMBC**	**HSQC**
Ring A, B, C				
2C		169.0	H-3C, H-2′B/H-6′	
3C	7.95; s	101.8		H-3C
4C		150.9	H-10D	
4aA		109.1	H-8A, H-6A, H-9D, H-3C	
5A		153.3	H-10D	
6A	7.13; d (1.70)	100.6		H-6A
7A		na		
8A	7.32; d (1.70)	101.0		H-8A
8aA		154.0	H-8A	
1′B		119.0	H-3C, H-2′B/H-6′B	
2′B/6′B	7.58; s	106.6	H-6′B/H-2′B	H-2′B/ H-6′B
3′B/5′B		148.9	OCH_3, H-2′B/ H-6′B	
4′B		144.0	H-2′B/H-6′B	
OCH_3	3.94; s	56.7		OCH_3
Ring D				
9D	7.03; d (5.33)	107.0	H-3C, H-10D	H-9D
10D	8.49; d (5.33)	159.6	H-9D	H-10D

s - singlet; d - doublet; dd – double doublet; na – not attributed

Table 11. ^{1}H and ^{13}C NMR data and HMBC and HSQC correlations of methylpyranodeoxypeonidin, determined in DMSO/TFA (90:10).

Position	**δ ^{1}H (ppm); *J* (Hz)**	**δ ^{13}C (ppm)**	**HMBC**	**HSQC**
Ring A, B, C				
2C		168.3	H-3C, H-2′B, H-6′B	
3C	7.78; s	101.1	H-9D	H-3C
4C		154.1	H-3C	
4aA		107.6	H-8A, H-6A, H-9D, H-3C	
5A		153.8	H-6A	
6A	7.13; d (1.50)	100.4	H-8A	H-6A
7A		167.3	H-6A, H-8A	
8A	7.32; d (1.50)	100.7	H-6A	H-8A
8aA		153.5	H-8A	
1′B		120.6	H-3C, H-5′B, H-2′B	
2′B	7.72; d (1.90)	111.5	H-6′B	H-2′B
3′B		148.8	OCH_3, H-2′B, H-5′B	
4′B		154.2	H-2′B, H-5′B, H-6′B	
5′B	7.06; d (8.43)	116.7		H-5′B
6′B	7.81; dd (1.90; 8.43)	123.5	H-2′B	H-6′B
OCH_3	3.91; s	56.3		OCH_3
Ring D				
9D	6.91; s	105.1	H-3C, CH_3	H-9D
10D		172.2	H-9D, CH_3	
CH_3	2.57; s	21.2	H-9D	CH_3

s - singlet; d - doublet; dd – double doublet

Chapter 5

Color Stability and Spectroscopic Properties of Deoxyvitisins in Aqueous Solution

Sousa, A., Cabrita, L., Araújo, P., Mateus, N., Pina F., de Freitas, V.

New J. Chem. 2014, 38(2), 539-544

In this work, the first author was responsible for the chemical synthesis of the tested compounds, in which he counted with the assistance from Paula Araújo in their purification. Spectroscopy experiments were performed by Luís Cabrita. The first author analyzed the experimental data and compared it with spectroscopy studies from analogous compounds available in the literature. He was always guided and advised by the rest of the authors.

Color Stability and Spectroscopic Properties of Deoxyvitisins in Aqueous Solution

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The color properties and stability of three types of 3-deoxyvitisins derived from peonidin (deoxyvitisin A, B and methyldeoxyvitisin) were studied by UV-Visible spectroscopy. Similarly to pyranoanthocyanins, the conjugated double bonds among pyranic rings C and D provide a higher electronic delocalization that prevents the nucleophilic attack of water at position 2 and the subsequent formation of the hemiketal and chalcone species. Consequently, besides flavylium cation (AH^+), neutral (A) and ionized bases (A^{n-}) have been identified by increasing pH, and the respective acidity constants were determined. The acidity constant values for the formation of the neutral quinoidal base ($pK_{a2(deoxyvit\ A)}$ = 4.8, $pK_{a1(deoxyvit\ B)}$ = 4.7 and $pK_{a1(methyldeoxyvitisin)}$ = 5.2) are slightly higher than the ones reported in the literature for their corresponding 3-glucosyl derivatives (vitisins). Given their higher stability, these pigments may be regarded as potential food colorants.

KEYWORDS: 3-deoxyvitisins, deoxyanthocyanidins, vitisins, anthocyanins, flavylium network, food colorants

INTRODUCTION

It is well known that the color of anthocyanins is greatly affected by the pH of the solution. The sequence of chemical reactions was correctly established by Brouillard et al. using temperature, pressure, and pH jump experiments: at very acidic pH, the red flavylium cation (AH^+) is the predominant species; when the pH is raised the flavylium cation immediately undergoes a proton transfer reaction, giving rise to the blue/purple quinoidal base (A), and simultaneously but more slowly the flavylium cation leads to the formation of colorless hemiketal (B) through the hydration reaction. The hemiketal compound further undergoes a tautomerization reaction to give the pale yellow cis-chalcone (Cc), which isomerizes to trans-chalcone (Ct) (Brouillard and Lang 1990).

3-Deoxyanthocyanidins are yellowish pigments commonly found in several food plants such as corn, black tea leaves and sorghum. Oppositely to anthocyanins, these pigments lack the 3-O-sugar moiety and show a significant increase in stability in slightly acidic solutions (Melo et al. 2007). It was recently demonstrated that at moderately acid pH values, while the predominant species in anthocyanins is the colorless hemiketal, in 3-deoxyanthocyanidins the equilibrium occurs between the species flavylium cation, *trans*-chalcone and quinoidal base. Moreover, the mole fraction of the base is approximately 50%, which contributes to the observed higher color intensity (Sousa, Petrov 2013). Several studies have also demonstrated the potential applications of these compounds for viable commercial food colorants, hair dyes, laser dyes, sensitizers for solar cells, molecular-level memory systems and health-promoting phytochemicals (Czerney et al. 1995, Cherepy et al. 1997, Roque et al. 2002, Awika et al. 2005, Shih et al. 2007).

On the other hand, pyranoanthocyanins differ from their genuine precursors (anthocyanins) in many aspects, especially in color. In general, they have a λ_{max} hypsochromically shifted (between 478 and 510 nm) conferring orange colors, except for portisins, which show a bathochromic shift in their λ_{max} values to more bluish hues around 580 nm (Mateus et al. 2003). Due to the additional pyranic ring, the color of these compounds is much more stable towards pH variations and bleaching by SO_2 in comparison to the parent anthocyanins, because it prevents the hydration reaction to give the hemiketal and the subsequent formation of the chalcone species (SarniManchado, Fulcrand 1996).

Although it has been generally accepted that anthocyanins are stable only at low pH values, it has been shown that, depending on the substitution pattern on the B-ring, some anthocyanin 3-glucosides lose 40-80% of their initial color intensity between pH 1 and pH 5-7, whereas pyranoanthocyanins practically do not change their color intensity (Fossen, Cabrita 1998, Cabrita et al. 2000, Oliveira et al. 2009, Oliveira, Petrov 2011, Oliveira, Mateus 2013).

Theoretically, designing deoxyanthocyanidins with a pyranic ring would result in compounds with further increased stability. Bearing this, we recently synthesized three types of 3-deoxypyranoanthocyanidins (deoxyvitisins) from the reaction between 3-deoxyanthocyanidins and pyruvic acid, vinyloxy-trimethylsilane or acetone-1,3-dicarboxylic acid (Sousa, Araujo 2013). In another study, a new natural pigment was

also isolated from red *Sorghum bicolor* with a deoxyvitisin-type structure, containing apigeninidin as a base unit (Khalil et al. 2010).

The aim of this work was to investigate the color stability and the network of chemical reactions occurring in aqueous solution upon pH variations for 3-deoxyvitisins, comparing the results with the model compound 4',7-dihydroxyflavylium and with values reported in the literature for corresponding vitisins.

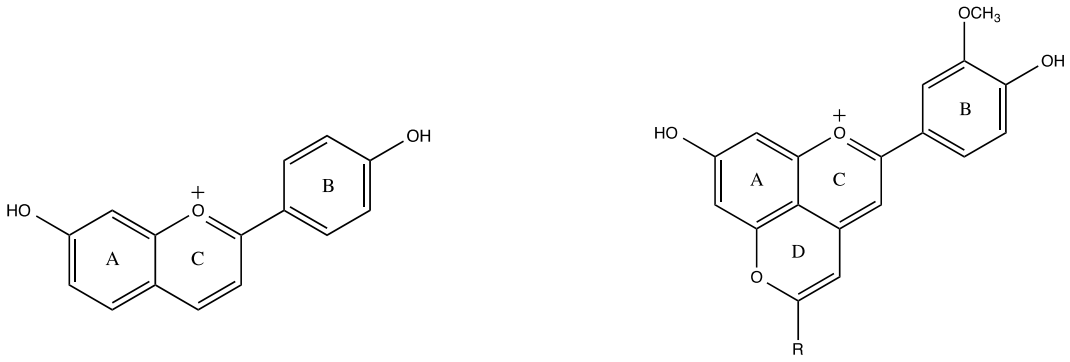

Fig. 46 - The model compound 4',7-dihydroxyflavylium and the 3-deoxyvitisins: methyldeoxyvitisin (R=CH_3), deoxyvitisin A (R=COOH) and B (R=H).

- MATERIALS AND METHODS

Synthesis of 3-deoxyvitisins and the model compound 4',7-dihydroxyflavylium. The synthesis of the deoxyvitisin compounds was followed according to the procedures described in the literature (Sousa et al. 2013). Initially, the synthesis of the reagent deoxypeonidin was performed by incubating phloroglucinol (8 mM) with coniferaldehyde (80mM) in a 12% ethanol aqueous solution, adjusted to pH 1.5 (Sousa et al. 2012). The deoxypeonidin was then incubated with the reagents pyruvic acid, vinyloxy-trimethylsilane and acetone-1,3-dicarboxylic acid in separate model aqueous solutions, under different conditions, as described elsewhere (He et al. 2006, Morata et al. 2007, Oliveira et al. 2009), to form the compounds deoxyvitisin A, B and methyldeoxyvitisin, respectively.

The formation of new compounds was followed by HPLC-DAD using a reversed phase C-18 (Merck®) column (250 x 4.6 mm i. d., particle size 5 µm), at 25 ºC. Solvents were (A) water/formic acid (95:5) and (B) acetonitrile. The elution gradient was performed using a L-2130 Merck® pump from 10 to 35% B for 55 min at a flow rate of 1.5 $mL.min^{-1}$. 4',7-dihydroxyflavylium was synthesized according to the method of Katritzky (Katritzky, Czerney 1998).

UV/Vis absorption spectra were recorded on a Varian Cary 100 Bio and Varian Cary 5000 spectrophotometers.

- RESULTS AND DISCUSSION

The most interesting feature of the three pigments studied in this work is their stability to the hydration reaction to give the hemiketal, hence preventing in this way the subsequent formation of *cis* and *trans* chalcones (incolor species). Identical behavior was reported for 4-methyl-7-hydroxyflavylium (Pina, Melo 1998), as well as for other pyranoanthocyanins (Oliveira et al. 2011).

Methyldeoxyvitisin (10-methylpyrano-3-deoxypeonidin). Fig. 48A shows the spectral variations of methyldeoxyvitisin occurring immediately after a pH jump from stock solutions at pH=1 to higher pH values (direct pH jumps). According to the spectral modifications and the chemical structure of the compound, two acid-base equilibria are predicted between the flavylium cation, the neutral quinoidal base and the ionized quinoidal base (Fig. 47). This is clearly visualized in the titration curves reported in Fig. 48B. Mathematical decomposition of the curves leads to the absorption spectra of each species, Fig. 48C.

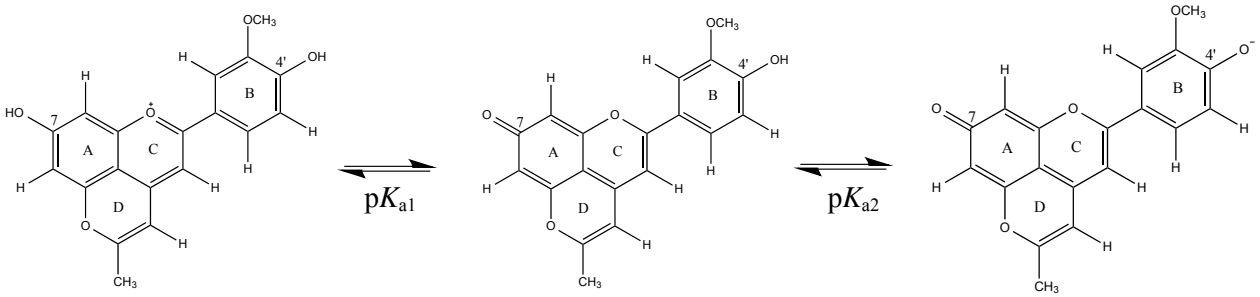

Fig. 47 - Equilibrium forms of 10-methyl-3-deoxyvitisin, see Table 12.

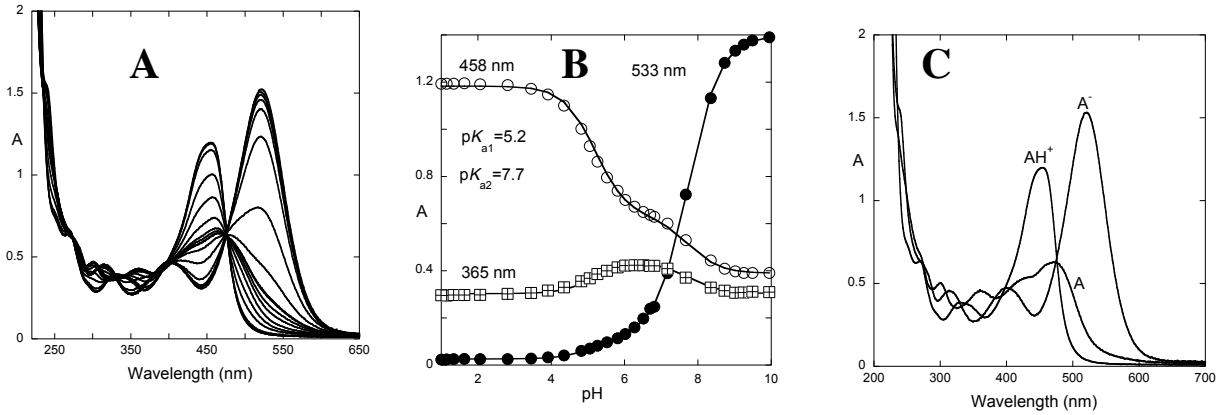

Fig. 48 - **A**- pH dependent absorption spectra of 10-methyl-3-deoxyvitisin 9.75x10^{-5} M (10% EtOH); **B**- Titration curves; fitting was achieved for pK_{a1}=5.2 and pK_{a2}=7.7; **C**- Absorption spectra of the three forms.

The behavior of methyldeoxyvitisin can be compared with the compound 4',7-dihydroxyflavylium when the absorption is collected immediately after a pH jump from pH=1 (flavylium cation) to higher pH values. Evidence that the first deprotonation in 4',7-dihydroxyflavylium takes place at the hydroxyl in position 7 was achieved by Jurd and Geissman (Jurd and Geissman 1963). Methyldeoxyvitisin is less acidic than 4',7-dihydroxyflavylium. This can be explained by the donor effect of the pyranic moiety increasing the electron density in the oxygen at position 7. The two acid-base equilibria are also in agreement with the values reported in the literature for methylpyranomalvidin-3-O-glucoside (pK_{a1} = 4.57; pK_{a2} = 8.23, Table 12) (Oliveira et al. 2011). The stability of the flavylium cation is also greater for the methyldeoxyvitisin due to the higher pK_{a1}, which can be explained by the absence of the 3-O-glucosyl and the 5'-methoxyl groups, when compared to the methylpyranoanthocyanin described in the literature.

Deoxyvitisin B (pyrano-3-deoxypeonidin). The chemical structure of deoxyvitisin B is similar to methyldeoxyvitisin but lacks the methyl substituent in position 10, Fig. 49 and Fig. 50.

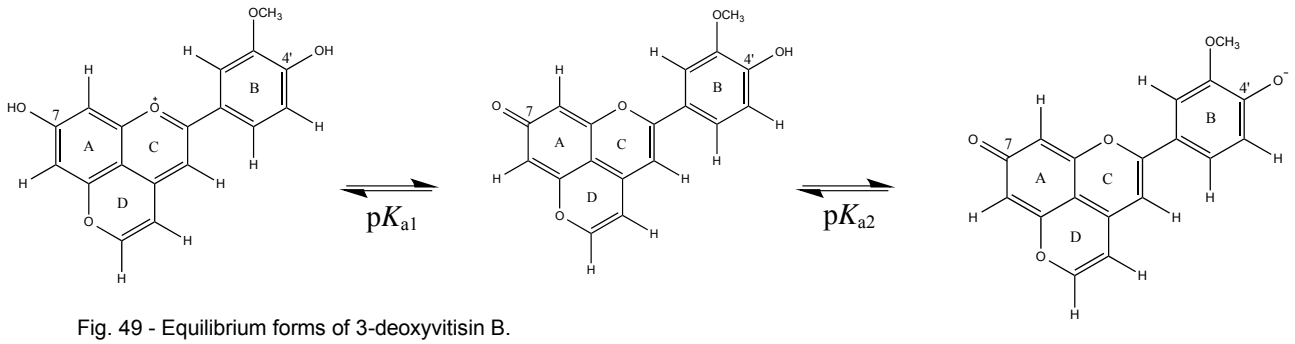

Fig. 49 - Equilibrium forms of 3-deoxyvitisin B.

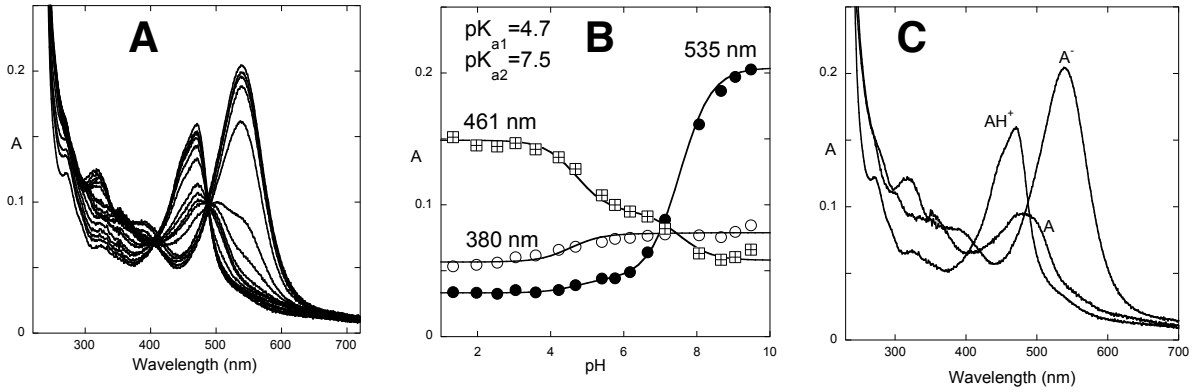

Fig. 50 - **A**- pH dependent absorption spectra of 3-deoxyvitisin B 9.98x10^{-5} M (10% EtOH); **B**- Titration curves; fitting was achieved for pK_{a1}=4.7 and pK_{a2}=7.5.

Regarding the first deprotonation, 3-deoxyvitisin B is more acidic than methyldeoxyvitisin. This could be explained by the donor effect of the methyl group in the latter. The second deprotonation, occurring at the 4'-hydroxyl, is influenced by the negative charge of the oxygen at position 7. In this case, the charge density is the main responsible for the increase between pK_{a1} and pK_{a2}, thus a similar pK_{a2} value is obtained for the four analyzed compounds reported in Table 12.

When comparing the results with the ones reported in the literature for visitin B (pK_{a1} = 4.40; pK_{a2} = 7.45, Table 12) (Oliveira et al. 2009), we observed again a higher pK_{a1} for 3-deoxyvitisin B than the corresponding vitisin B. This is similar and can be explained as above in the case of 10-methyl-3-deoxyvitisin and the corresponding methylpyranoanthocyanin.

Deoxyvitisin A (10-carboxypyrano-3-deoxypeonidin). In the case of the compound deoxyvitisin A, three pK_a's are predicted, Fig. 51. The pH dependent

absorption spectra of this compound are shown in Fig. 52. The titration shown in Fig. 52B is compatible with the expected three pK_a's. In a previous work (Gavara, Laia 2010, Gago, Petrov 2012) it was shown that the absorption spectra of flavylium derivatives containing a carboxylic substituent, is only slightly dependent on the protonation state of the carboxylic acid/carboxylate. Fig. 52C indicates a great similarity between the absorption spectra of the flavylium cation possessing the carboxylic acid or the carboxylate. Furthermore, in a previous work it was demonstrated by NMR that the carboxylic substituent of vitisin A was the first to be deprotonated due to the differences in the displacement of chemical shifts for the protons in the aromatic region (Oliveira et al. 2013). On this basis, the second deprotonation can be assigned to position 7 and the third one to 4' as compared to the other 3-deoxyvitisins and flavylium derivatives in general. When looking at the results reported in the literature for vitisin A (pK_{a1} = 1.18; pK_{a2} = 4.42; pK_{a3} = 7.78, Table 12), a pattern similar to the other 3-deoxyvitisins can be observed, exhibiting a slightly higher acidity constant for the hydroxyl in position 7 than the corresponding vitisin pigments.

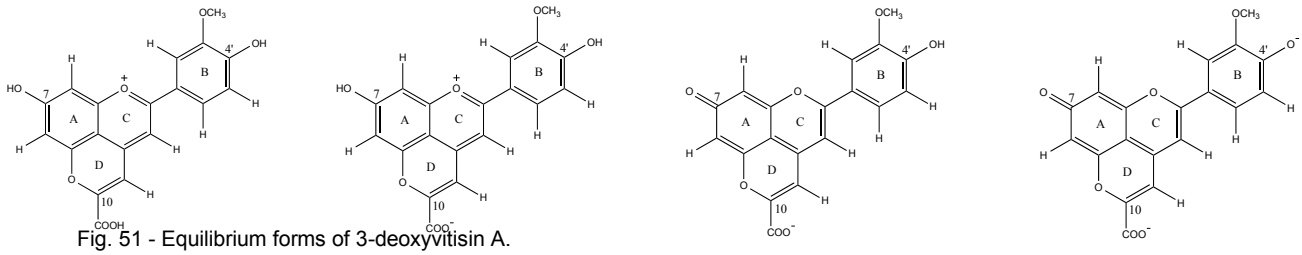

Fig. 51 - Equilibrium forms of 3-deoxyvitisin A.

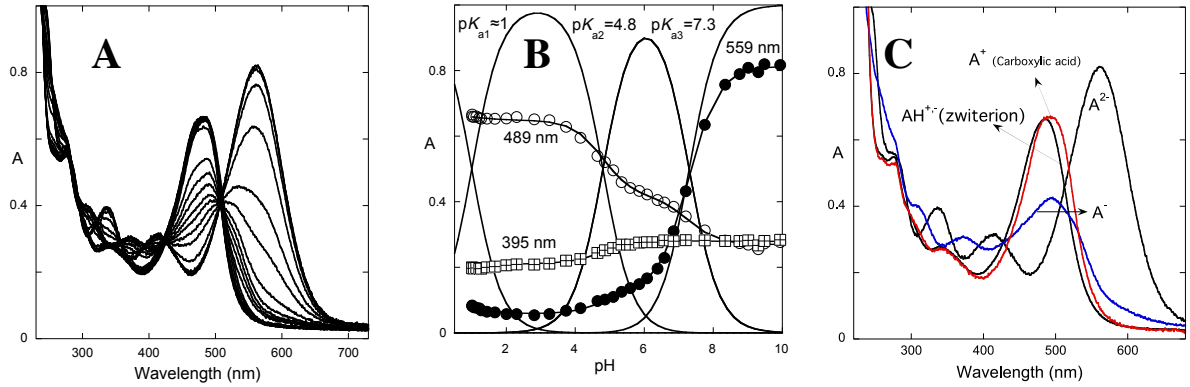

Fig. 52 - **A**- pH dependent absorption spectra of 3-deoxyvitisin A 1.16x10^{-4} M (10% EtOH); **B**- Titration curves; fitting was achieved for pK_{a1}≈1 and pK_{a2}=4.8, pK_{a3}=7.3; **C**- Absorption spectra of the four species obtained by mathematical decomposition. The absorption spectra at pH= 2.9, 6.1 and 10 correspond practically to the species $AH^{+\cdot}$ (zwitterion), A^- and A^{2-}, respectively. At pH=1.0 there is a mixture (1:1) of the zwitterion and the flavylium cation (possessing the carboxylate unit protonated) permitting to obtain the spectrum of this last.

Table 12. Dissociation constants (pK_a's).

Compound	pK_{a1}	pK_{a2}	pK_{a3}
4',7-DiOH	4.1 (Pina et al. 2012)	7.2	
Methylpyranodeoxypeonidin	5.2	7.7	
Deoxyvitisin B	4.7	7.5	
Deoxyvitisin A	≈1*	4.8	7.3
Literature			
Methylpyranomalvidin-3-O-glc (Oliveira et al. 2011)	4.57	8.23	
Vitisin B (Oliveira et al. 2009)	4.40	7.45	
Vitisin A (Oliveira et al. 2013)	1.18	4.42	7.78

*Deprotonation of the carboxylic acid in position 10

- CONCLUSION

The color stability and chemical equilibria of 3-deoxypyranoanthocyanidins in aqueous solution has been thoroughly explained and described by UV-Visible spectroscopy. It was confirmed that like pyranoanthocyanins and oppositely to anthocyanins, 3-deoxypyranoanthocyanidins do not show hydration reactions in aqueous solutions, but undergo deprotonation reactions at higher pH values.

The acidity constants for the three 3-deoxypyranoanthocyanidins studied have been determined and compared to a model compound and to pyranoanthocyanins reported in the literature. Deoxypyranoanthocyanidins were found to be less acidic, thus more stable, than the corresponding pyranoanthocyanin 3-O-glucosides.

The first deprotonation is relatively dependent on the substitution pattern of the compounds, while the second one (deprotonation of the hydroxyl substituent in position 4') is more or less invariant. We were able to determine the ionization constant of the carboxylic acid substituent of deoxyvitisin A which is in good agreement with what was reported for its glycosylated derived compound, vitisin A.

Given the fact that the loss of color in solution of these compounds is lower than anthocyanins they may be regarded as potential commercial food colorants. However, further studies should be performed in order to test their stability to other conditions (temperature, sulfites, etc) as well as to study their antioxidant properties.

- SUPPLEMENTARY MATERIAL

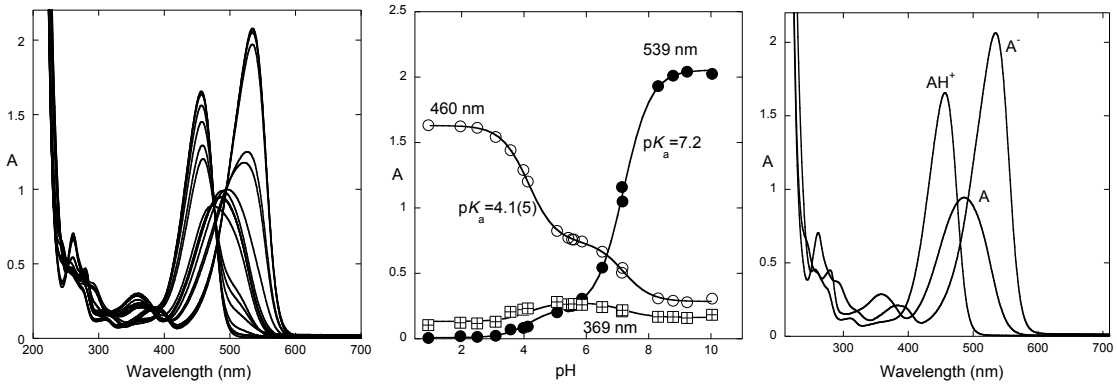

Fig. 53 - **A**- pH dependent absorption spectra of 4',7-dihydroxyflavylium; **B**- Titration curves; fitting was achieved for pK_{a1}=4.15 and pK_{a2}=7.2; **C**- Absorption spectra of the three species obtained by mathematical decomposition.

- AKNOWLEDGEMENTS

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Chapter 6

Evidence for Copigmentation Interactions between Deoxyanthocyanidin Derivatives (Oaklins) and Common Copigments in Wine Model Solutions

Sousa, A., Araújo, P., Cruz, L., Brás, N. F., Mateus, N., de Freitas, V.

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In this work, the first author was responsible for all the experimental activities with the exception of molecular dynamic simulations, which were performed by Natércia Brás. He also counted with the help of Paula Araújo in the purification of oaklins and with Luís Cruz in the copigmentation experiments and data interprentation. Guidance and advice were also obtained from the rest of the authors, regarding project planning and data interpretation.

Evidence for Copigmentation Interactions between Deoxyanthocyanidin Derivatives (Oaklins) and Common Copigments in Wine Model Solutions

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The aim of this study was to investigate interactions possibly taking place in red wine between oaklins, that are deoxyanthocyanidin derivatives (guaiacylcatechin-pyrylium and syringylcatechin-pyrylium) and several copigments: catechin (CP1), epicatechin (CP2), chlorogenic acid (CP3), epigallocatechin (CP4) and procyanidin B3 (CP5). The results show that oaklins, like common anthocyanins, also present copigmentation interactions that further stabilize the flavylium cation in hydroalcoholic solutions. Molecular dynamics simulations were also performed to interpret the binding data, to specify the relative arrangement of the pigment and copigment molecules within the complexes, and to interpret their absorption properties in the visible range.

KEYWORDS: oaklins; copigments; deoxyanthocyanidins; anthocyanins; aldehydes; flavylium network; food colorants; wine aging; molecular dynamics

- INTRODUCTION

Oaklins are catechinpyrylium compounds formed in red wines aged in oak barrels and result from the reaction between catechin and wood cinnamic aldehydes. They present a brick-red color and oppositely to anthocyanins, these pigments do not possess a glycosyloxy substituent at C3 of the flavylium core and are thus classified as deoxyanthocyanidins (de Freitas et al. 2004, Sousa et al. 2005).

Despite the thermodynamic tendency of anthocyanins to form colorless hemiketals, natural colors expressed by anthocyanins are fairly stable, which evidences naturally occurring stabilization mechanisms. Indeed, copigmentation has been one of the most important mechanisms described in the literature to stabilize the colored forms. Copigmentation mainly refers to interactions between colorless phenols (copigments)

and the planar polarizable nuclei of anthocyanins' colored forms. The association is promoted by the hydrophobic effects, mainly dispersive π−π stacking interactions between the polarizable orbitals of the aromatic rings (Galland, Mora 2007, Gonzalez-Manzano, Duenas 2009, Yoshida, Mori 2009, Cruz, Bras 2010).

In a previous investigation it was concluded that when compared to common wine anthocyanins in which the hemiketal B (incolor) is the major species at wine pH (~3.5), the equilibrium of oaklins involves essentially the flavylium cation AH^+, the quinoidal base A, and the *trans*-chalcone Ct and the % of hemiketal is very low. The behavior is similar to simpler deoxyanthocyanidins as described in the literature. Furthermore, the pK'_a values obtained for oaklin compounds were higher than the ones reported for malvidin-3-glucoside, meaning that the flavylium cation is more stable in the former. The loss of color in solution is thereby greatly diminished in oaklin compounds when compared to other anthocyanins, not only because the pK'_a is greater but also because the mole fraction of the base is substantially higher at higher pH values. Nevertheless, the effects of copigmentation cannot be underestimated when analyzing color changes in wine solutions (Sousa et al. 2013).

It is well known the importance and contribution of the copigmentation effect on anthocyanin pigments (Asen, Stewart 1971, 1972, Mazza and Brouillard 1990). It was only recently that the copigmentation interactions between deoxyanthocyanidins with some phenolics were studied (Awika 2008). The aim of this work was thus to study the copigmentation interactions possibly occurring between the oaklin compounds guaiacylcatechinpyrylium (GCP) and syringylcatechinpyrylium (SCP) with common copigments such as catechin, epicatechin, chlorogenic acid, epigallocatechin, procyanidin B3 and determine the respective binding constants. The relationship between copigmentation ability and the structure of the complexes was also evaluated.

- MATERIALS AND METHODS

Samples. (+)-Catechin (C), (−)-epicatechin (EC) and chlorogenic acid were purchased from Sigma-Aldrich (Madrid, Spain). (−)-Epigallocatechin (EGC) was purchased from Biopurify Phytochemicals Ltd. (Sichuan, China). Procyanidin B3 (PCB3) was extracted from barley and isolated according to the procedures described elsewhere (Dvorakova, Moreira 2008, Teixeira, Cruz 2013). The pigments guaiacylcatechinpyrylium (GCP) and syringylcatechinpyrylium (SCP) were obtained through the chemical synthesis between catechin and coniferaldehyde or sinapaldehyde, respectively, according to the procedure described elsewhere (Sousa et al. 2013).

Copigmentation. All solutions used were prepared in a citrate buffer solution (0.2 M) with 10% ethanol at pH 3.5, and the ionic strength was adjusted to 0.5 M by the addition of sodium chloride. Each pigment/copigment solution was prepared by mixing a volume of pigment (10^{-4} M) solution with an aliquot of copigment solution to give the required pigment/copigment molar ratio of 1:0, 1:1, 1:5, 1:10, 1:20, 1:30, 1:40. Each experiment was performed in triplicate. All of the solutions were left to equilibrate for 30 min before spectroscopic measurements. For GCP with the copigment epigallocatechin, the absorbance values were collected at the wavelength (λ_{max} 498 nm) of the isosbestic point of the flavylium cation (10^{-4} M) and its copigmentation complex (GCP/copigment molar ratio=1:40). This parameter was determined in strongly acidic solutions (1 M aqueous HCl, pH ≈0) in which the flavylium ion is the sole anthocyanin form. However, for the rest of the copigments and for the pigment SCP, an isosbestic point was not observed, hence the absorbance values were collected at the maximum absorption wavelength of free pigment (GCP/SCP) at pH 3.5 (λ_{max} 485 nm for GCP and 500 nm for SCP).

UV−Visible Spectroscopy. UV−visible spectra were recorded on a Bio-Tek Power Wave XS spectrophotometer at a constant temperature of 25 °C from 360 to 830 nm (1 nm sampling interval) using a 1 cm path length cell.

Data Analysis. The curve fittings were carried out using the software program MicroMath (Salt Lake City, UT, USA). Curve fittings were achieved through a least-squares regression method. Statistical analysis reported standard deviations and correlation coefficients.

Molecular Dynamic Simulations. The initial geometries of the pigments (GCP and SCP) and copigments (catechin, epicatechin, epigallocatechin, B3 and chlorogenic acid) molecules were built with the GaussView software (Gaussian). To calculate the optimized geometries and electronic properties for the subsequent parameterization of these compounds, the Gaussian 09 suite of programs (Frisch 2009) was used to perform restricted Hartree-Fock calculations (RHF), with the 6-31G(d) basis set. Atomic charges were further recalculated using the RESP algorithm (Bayly, Cieplak 1993). This methodology was chosen for its consistency with that adopted in the parameterization process of AMBER 10.0 simulation package (Case 2008). MD (Molecular Dynamic) simulations were performed with GAFF (generalized amber force field for small organic molecules) (Wang, Wolf 2004) and the TIP3P model for the solute and water, respectively. Explicit solvation was included as a rectangular box with a 12Å distance between the box faces and any atom of the compounds. One counter-ion (Cl^-) was employed to neutralize the positive charge of each system, which has a size of

approximately 5,700 atoms. All complex geometries were minimized in two stages: first, the solute was kept fixed and only the position of the counter-ion and water molecules was minimized (500 steps using the steepest descent algorithm and 1,500 steps carried out using conjugate gradient); second, the full system was minimized (1,000 steps using the steepest descent algorithm and 2,000 steps carried out using conjugate gradient). Subsequently, an MD simulation of 100 ps at constant volume and temperature, and considering periodic boundaries conditions was run, followed by 30 ns MD simulation with the NPT ensemble, in which Langevin dynamics was used (collision frequency of 1.0 ps^{-1}) to control the temperature at 303.15 K (Izaguirre, Catarello 2001). All simulations were carried out using the PMEMD module, implemented in the Amber 10.0 simulation package (Case 2008). Bond lengths involving a hydrogen atom were constrained using the SHAKE algorithm, and the equations of motion were integrated with a 2 fs time-step using the Verlet leapfrog algorithm (Ryckaert, Ciccotti 1977). The Particle-Mesh Ewald (PME) method (Essmann, Perera 1995) was used to include the long-range interactions, and the nonbonded interactions were truncated with a 10Å cutoff. The MD trajectory was saved every 2 ps and the MD results were analyzed with the PTRAJ module of AMBER 10.0 (Case 2008).

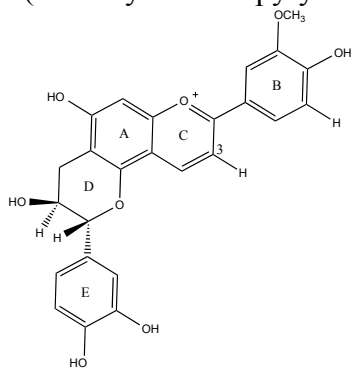
Calculation of Relative Binding Free Energies ($\Delta\Delta G_{binding}$). The MM-PBSA script (Molecular Mechanics−Poisson−Boltzmann Surface Area) (Kollman, Massova 2000, Massova and Kollman 2000, Huo, Massova 2002) as implemented in Amber 10.0 simulations package (Case 2008) was used to calculate the binding free energies ($\Delta G_{binding}$) for all complexes. A series of 150 geometries was extracted every 100 steps of each simulation. The internal energy (bond, angle, and dihedral), the electrostatic and the van der Waals interactions were calculated using the Cornell et al. force field (Cornell, Cieplak 1995) with no cutoffs. The electrostatic solvation free energy was calculated by solving the Poisson−Boltzmann equation with the PBSA program. The nonpolar contribution to the solvation free energy due to van der Waals interactions between the solute and the solvent and cavity formation was modeled as a term that is dependent on the solvent accessible surface area of the molecule. As these compounds possess similar structures and binding modes, the relative binding energies ($\Delta\Delta G_{binding}$) were calculated with respect to the most stable complex.

- RESULTS AND DISCUSSION

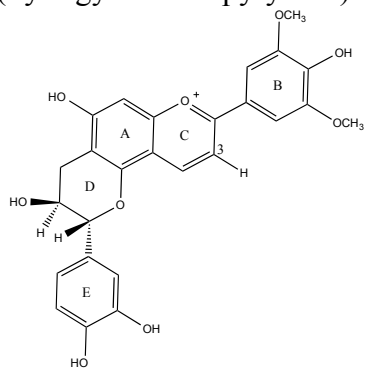
The association of the oaklins GCP (guaiacylcatechinpyrylium) and SCP (syringylcatechinpyrylium) with the copigments selected (Fig. 54) was quantitatively

evaluated through the determination of the copigmentation binding constants (K_{CP}) for each pigment-copigment pair.

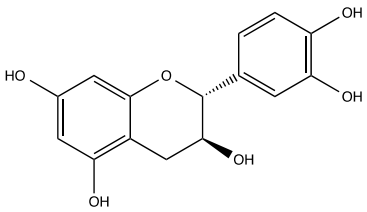
GCP (Guaiacylcatechinpyrylium)



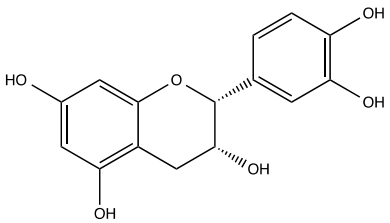
SCP (Syringylcatechinpyrylium)



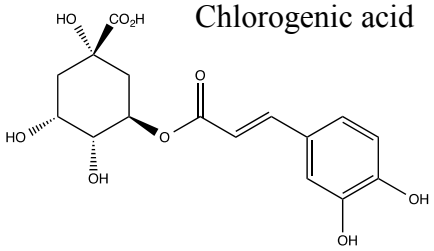
Catechin



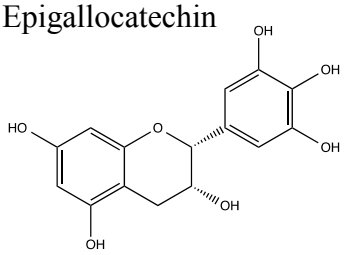
Epicatechin



Chlorogenic acid



Epigallocatechin



Procyanidin B3

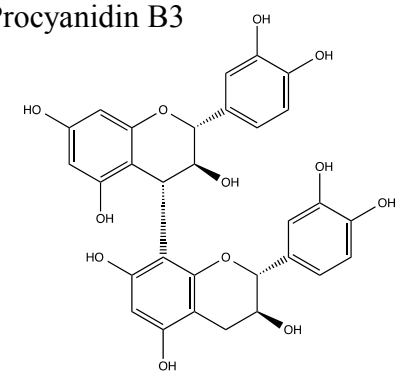


Fig. 54 - Chemical structures of the deoxyanthocyanidin derivatives - oaklins (GCP and SCP) and the copigments investigated in this work.

To minimize self-association effects in the intermolecular copigmentation studies, a low concentration of pigment (1×10^{-4} M) was used. Although ethanol largely reduces the copigmentation effect (Dangles and Brouillard 1992), its inclusion in the experimental conditions was crucial to prevent the precipitation during the spectroscopic analysis of the pigment and copigment compounds due to their low solubility in water.

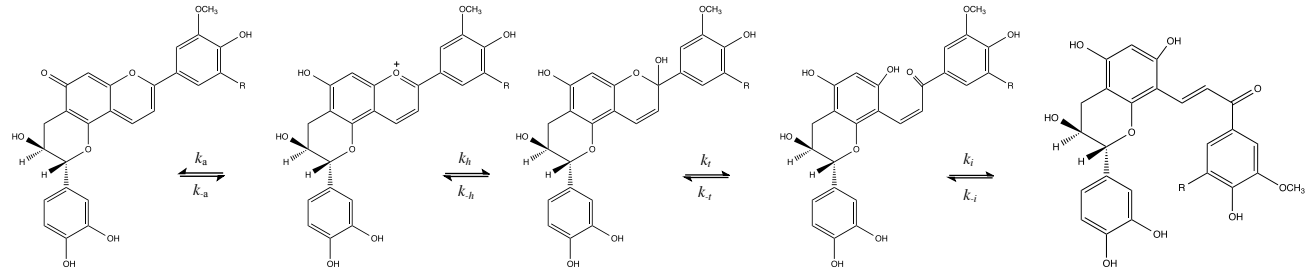


Fig. 55 - Network of chemical reactions of oaklins (R=OCH_3, SCP; R=H, GCP).

Copigmentation interactions occurring in oaklins. In a very acidic medium, only the flavylium cation is present and the visible absorbance is

$$A_0 = \varepsilon_{AH^+} C$$

where C is the total pigment concentration, and can be approximated to $C = [AH^+]$.

Oppositely to anthocyanins, in which the contribution of quinonoidal bases (A) is negligible in mildly acidic medium (pH 3.5) and the hemiketal is the predominant species, in deoxyanthocyanidins, particularly oaklins, the equilibrium plateau occurs between the flavylium cation, the neutral quinoidal base and the *trans*-chalcone, with the hemiketal and the *cis*-chalcone being transient species (Sousa et al. 2013). Copigmentation of the quinoidal bases could make a contribution, as those forms are also prone to stacking interactions with copigments. However, as the quinoidal bases and their copigmentation complexes absorb the visible light at higher wavelengths than their flavylium counterparts, no specific contribution is expected in the measured wavelength range (480-500 nm) (Haslam and Lilley 1988, Pina 1998). Copigmentation of quinoidal bases also promotes a high bathochromic shift (which is not observed in our case), especially with purine copigments like caffeine and theophylline (Pina et al. 2012).

Bearing this in mind, the total concentration of the pigment for deoxyanthocyanidins at mildly acidic solutions can be represented by (Malien-Aubert, Dangles 2002, Awika 2008)

$$C = [AH^+] + [A] + [B] + [C_c] + [C_t]$$

$$= [AH^+] + [A] + [B']$$

where B′ is the pool of colorless forms in equilibrium.

The apparent thermodynamic constant for the overall reactions (Fig. 55) taking place is thus given by

$K'_a = K_a + K_h + K_h K_t + K_h K_t K_i$

Being so, one can also write

$$C = \frac{AH^+}{1 + \frac{K'_a}{H_3O^+}}$$

Oppositely to common anthocyanins in which $A_0 = \frac{\varepsilon_{AH^+} AH^+}{1 + \frac{K_h}{H_3O^+}}$, from the equations above, and in the absence of the copigment one easily obtains

$$A_0 = \frac{\varepsilon_{AH^+} C}{1 + \frac{K'_a}{H_3O^+}} \qquad \text{(eq 1)}$$

In the presence of the copigment, and assuming a 1:1 stoichiometry for the complex, the visible absorbance at pH 3.5 becomes

$A = \varepsilon_{AH^+} C + \varepsilon_{AHCP^+} [AHCP^+]$

We thus have

$$A = \frac{\varepsilon_{AH^+} C \{1 + r K_{CP}[CP]\}}{1 + \frac{K'_a}{H_3O^+}] + K_{CP}[CP]} \qquad \text{(eq 2)}$$

with $r = \frac{\varepsilon_{AHCP^+}}{\varepsilon_{AH^+}}$

By combining equation 1 and 2, *A* can also be expressed as (Malien-Aubert et al. 2002, Teixeira et al. 2013)

$$A = \frac{\epsilon_{AH^+} C_0 [1 + r K_{CP} [CP]_1}{\frac{a}{r-a} + \frac{1}{(r-a) K_{CP}} \times \frac{1}{[CP]_1}} + A_0 \qquad \text{(eq 3)}$$

where [CP] is the total concentration of the pigment, $a = \frac{1}{1 + \frac{K'_a}{10^{-pH}}}$, A and A_0 represent the visible absorbance of the pigment in the presence and absence of copigment, respectively, and r is the ratio of the molar absorption coefficient of the complex to that of the free flavylium ion. Since r is the ratio between the molar absorption coefficient of the copigmentation complex and the free flavylium ion, r=1 by definition at the isosbestic point (determined at pH≈0 to ensure total conversion of the pigment into the flavylium form). Surprisingly, it was only possible to determine the isosbestic point with the pair GCP/epigallocatechin. Indeed, for the rest of the compounds, at pH≈0, an unexpected hyperchromic effect of the pigment-copigment complex was observed instead of a bathochromic shift, which is typical of copigmentation interactions. In these cases, the maximum absorption wavelength of the complex was selected for the calculations and parameter r was estimated to be the ratio between the absorbance of the copigmentation complex and the free flavylium form at this wavelength (Table 13 and Table 14).

Table 13. Absorbance values for the GCP/copigment complexes.

	r = 1.06	**r = 1.01**	**r = 1.01**	**r = 1.00**	**r = 1.09**
Pigment / copigment	**Catechin**	**Epicatechin**	**Chlorogenic Acid**	**Epigallocatechin**	**Procyanidin B3**
molar ratio	**(λ_{max} 485 nm)**	**(λ_{max} 485 nm)**	**(λ_{max} 485 nm)**	**(λ_{max} 498 nm)**	**(λ_{max} 485 nm)**
1:0 (A_0)	0.149	0.168	0.180	0.132	0.138
1:5	0.154	0.182	0.189	0.141	0.147
1:10	0.161	0.192	0.194	0.147	0.155
1:20	0.170	0.208	0.208	0.160	0.168
1:30	0.184	0.227	0.226	0.174	0.178
1:40	0.192	0.241	0.242	0.188	0.188

Table 14. Absorbance values for the SCP/copigment complexes at the maximum wavelength (λ_{max} 500 nm).

	r = 1.23	r = 1.30	r = 1.28	r = 1.38	r = 1.15
Pigment / copigment molar ratio	**Catechin**	**Epicatechin**	**Chlorogenic Acid**	**Epigallocatechin**	**Procyanidin B3**
1:0 (A_0)	0.152	0.180	0.179	0.154	0.153
1:5	0.157	0.196	0.184	0.166	0.161
1:10	0.163	0.207	0.193	0.180	0.171
1:20	0.174	0.236	0.207	0.201	0.182
1:30	0.189	0.253	0.223	0.215	0.191
1:40	0.200	0.272	0.233	0.237	0.202

It is observed an absorbance increase with the copigment concentration, which reflects a preferential association of the copigment to the flavylium cation and the subsequent shift of the hydration toward the colored forms.

For each copigment, a plot of absorbance as a function of wavelength was presented and the typical behavior of copigmentation phenomena was perceived: increase of absorbance (hyperchromic effect) and a slight batochromatic shift of λ_{max} as the copigment concentration increased. Fig. 56 presents the absorption spectra of free GCP and GCP/epigallocatechin complex obtained at several molar ratios (**A**) and the corresponding mathematical treatment to determine K_{CP} according to eq 3 (**B**).

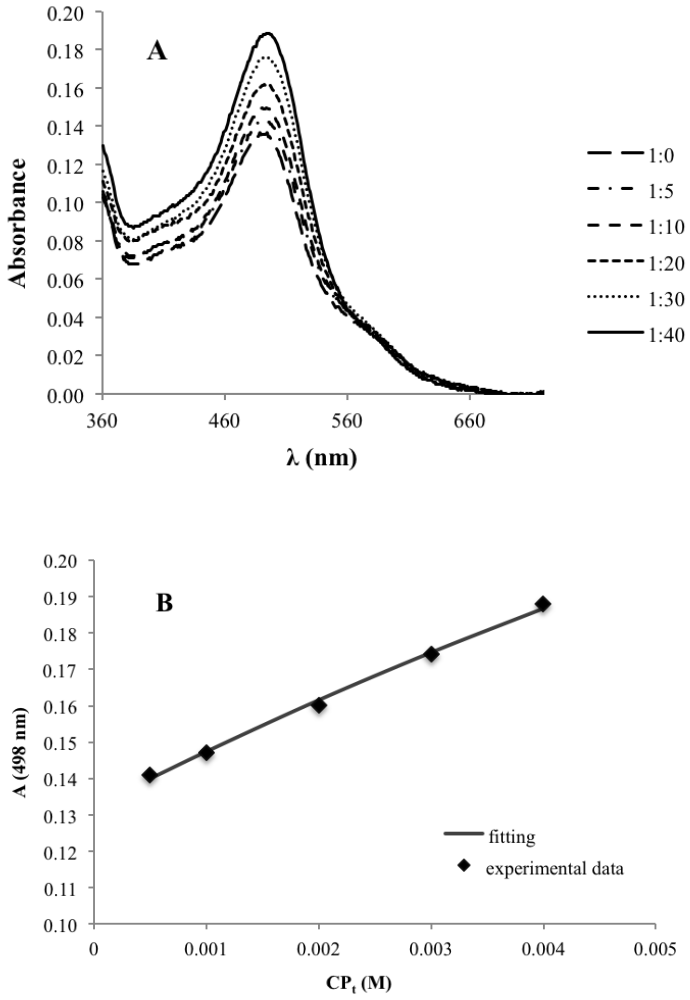


Fig. 56 - (**A**) Absorption spectra of GCP (0.1 mM) in the presence of epigallocatechin in a pH 3.5 phosphate buffer with 10% ethanol at 25 °C. Pigment/copigment molar ratios: 1:0, 1:5, 1:10, 1:20, 1:30, 1:40. (**B**) Plot of the visible absorbance at 498 nm as a function of copigment concentration; experimental points (◆) and theoretical curve (−) from a curve fitting according to eq 3 (pK'_a set at 3.05).

Equation 3 was used for the curve fitting of the experimental data A = f([CP]) to achieve the optimized values for KCP as the sole adjustable parameter. Statistical analysis gave good correlation coefficients and standard deviations for the copigmentation constants (KCP) (Table 15).

Table 15. Copigmentation constants for the oaklin/copigment complexes.

	GCP		SCP		Oenin	Malvin
Copigment	**K_{CP}**	**R^2**	**K_{CP}**	**R^2**	**K_{Cp}**	**K_{Cp}**
Catechin	101 (±2)	0.997	96 (±3)	0.998	136 (±4)*	-
Epicatechin	176 (±5)	0.999	165 (±4)	0.998	99 (±2)	-
Chlorogenic Acid	125 (±4)	0.995	90 (±2)	0.998	-	350**
Epigallocatechin	165 (±3)	0.998	152 (±4)	0.995	177 (±7)	-
Procyanidin B3	130 (±4)	0.998	117 (±5)	0.996	48 (±2)	-

*(Teixeira et al. 2013)

**(Nave, Bras 2012)

The highest copigmentation binding constants were observed for the complexes with the copigment epicatechin with both pigments (GCP and SCP). The two pigments also followed a similar copigmentation pattern, with the following highest copigmentation constants observed in decreased order for epigallocatechin and procyanidin B3. For GCP, the lowest two were observed in decreased order for chlorogenic acid and catechin, whereas for SCP the decreased order was catechin and chlorogenic acid.
When comparing the copigmentation constants obtained for these deoxyanthocyanidin derivatives with the ones reported for anthocyanins, we observed similar results regarding their order of magnitude. However, we found some interesting results for some copigments, such as epicatechin and procyanidin B3, which presented higher binding constants than what was expected for anthocyanins (Brouillard, Wigand 1991, Nave et al. 2012, Teixeira et al. 2013).

Computational Studies of pigment-copigment complexes. To better characterize the copigmentation behaviour of each complex, computational studies were performed on copigment molecules (catechin, epicatechin, epigallocatechin, B3 and chlorogenic acid) complexed with both GCP and SCP pigments. Molecular dynamics simulations of 30 ns were carried out for these 10 distinct complexes, which allowed the respective conformational space to be sampled in order to identify several conformations for each copigmentation complex studied. A similar protocol was used in previous studies in the literature (Cruz et al. 2010, Nave et al. 2012, Teixeira et al. 2013). The relative binding free energies obtained by the MM-PBSA approach (Kollman et al. 2000, Massova and Kollman 2000, Huo et al. 2002) are in total agreement with the experimental results (Table 16), which suggests that the MD simulations provide an accurate portrait for conformational analysis. Although the theoretical $\Delta\Delta G_{binding}$ values fit qualitatively with the experimental values, the quantitative differences are ten times

higher, which could be due to some approximations within the computational method. These results suggest that both GCP and SCP pigments have the ability to interact with the copigment compounds tested. Considering the copigmentation group with the GCP as pigment, the $\Delta G_{binding}$ energy of GCP-epicatechin complex is the most negative value, followed by GCP-epigallocatechin, GCP-B3, GCP-cholorogenic acid and GCP-catechin. In relation to the group with the SCP as pigment, the ranking obtained for the binding free energies is SCP-epicatechin > SCP-epigallocatechin > SCP-B3 > SCP-catechin > SCP-cholorogenic acid. In fact, this data indicates that the GCP/SCP-epicatechin and GCP/SCP-epigallocatechin complexes display higher stability and their formation is thermodynamically favored when compared to the other complexes. However, the maximum experimental and theoretical ΔΔGbinding values (0.36 and 2.73 kcal/mol, respectively) reveal a higher stability for all copigmentation complexes.

Table 16. Relative binding free energies of the copigmentation complexes.

Pigment	Copigment	Theoretical $\Delta\Delta G_{binding}$ (kcal/mol)	Experimental $\Delta\Delta G_{binding}$ (kcal/mol)[a]
GCP	Epicatechin	0.00	0.00
	Epigallocatechin	0.65	0.04
	B3	1.39	0.18
	Chlorogenic acid	1.77	0.20
	Catechin	3.19	0.33
SCP	Epicatechin	0.00	0.00
	Epigallocatechin	0.36	0.05
	B3	1.10	0.20
	Catechin	1.77	0.32
	Chlorogenic acid	2.73	0.36

[a]Experimental values calculated from Table 15 using $\Delta\Delta G = RT \ln(K_{Pigment:Epicatechin}/K_{Pigment:X})$

Fig. 57 shows the closest geometries to the average structures for the 10 GCP-copigment and SCP-copigment complexes. As expected, the formation and stabilization of each complex is ensured by the establishment of hydrophobic interactions between the polyphenolic rings, as well as hydrogen bonds with the hydrophilic hydroxyl polar

groups of both compounds. According to the literature, the formation of copigmentation complexes is driven by van der Waals interactions between the large planar surfaces of the pigment and copigment and the concomitant release of high-energy water molecules from the solvation shells (hydrophobic effect).

Although the copigmentation systems studied here have large number of degrees of freedom, the geometries displayed in Fig. 57 show several optimized π-stacking arrangements. Hence, the proximity between aromatic planar surfaces of the pigment and copigment molecules should reflect the stability of each complex. The interplanar distances between the CAD nucleus, B and E rings of GCP/SCP and the aromatic and benzopyrylium rings of each copigment were calculated along each MD simulation. Table 17 shows the minimal distances obtained. The results show that the benzopyrylium and B rings of epicatechin and cholorogenic acid molecules are the closest planes to the CAD nucleous of GCP (4.95 Å and 4.65 Å, respectively). In addition, epicatechin is the copigment that has the nearest planes to the B and E rings of GCP (4.82 Å and 7.11 Å, respectively). Relatively to the copigmentation complexes with SCP, the epicatechin, B3 and catechin show the minimal distances to the CAD, B and E planes of this pigment (5.48 Å, 4.87 and 5.84 Å, respectively). Since intermolecular distances of about 4 Å are consistent with van der Waals contacts, this data reveals that the shortest copigment-pigment distances were obtained for the complexes with the epicatechin copigment, which agrees with their highest stability constants (K).

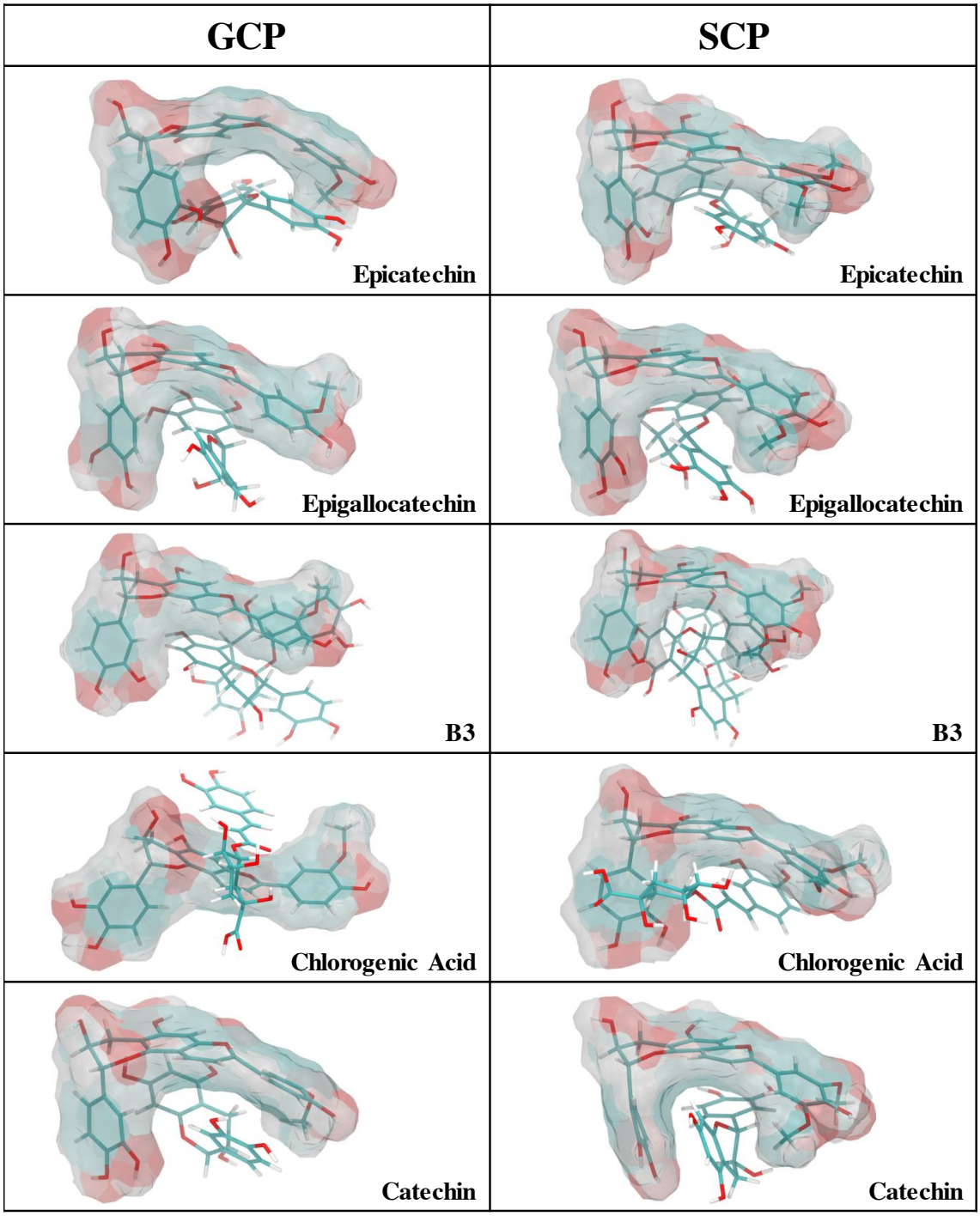


Fig. 57 - Closest geometries to the average structures of the GCP/SCP-epicatechin, GCP/SCP-epigallocatechin, GCP/SCP-procyanidin B3, GCP/SCP-cholorogenic acid and GCP/SCP-catechin complexes. Carbon, oxygen and hydrogen atoms are coloured in blue, red and white, respectively. The charged pigment is depicted in a surface representation.

Table 17. Average minimal distances between approximately planar surfaces of the GCP/SCP pigments and copigment molecules in each copigmentation complex.

Pigment	Copigment	Average minimal distance (Å)		
		CAD	B	E
GCP	Epicatechin	4.95 (AC)	4.82 (B)	7.11 (AC)
	Epigallocatechin	5.77 (AC)	5.97 (AC)	7.94 (B)
	B3	5.81 (AC')	5.52 (B')	8.35 (AC')
	ChAcid	4.65 (B)	6.11 (B)	9.21 (B)
	Catechin	5.31 (B)	6.56 (B)	8.44 (B)
SCP	Epicatechin	5.28 (B)	6.37 (B)	6.60 (AC)
	Epigallocatechin	5.65 (AC)	5.71 (AC)	7.18 (B)
	B3	5.37 (AC)	4.87 (B)	6.83 (AC)
	Catechin	5.48 (AC)	5.69 (B)	5.84 (AC)
	ChAcid	5.78 (B)	5.11 (B)	7.59 (A)

According to the structures of the 10 complexes shown in Fig. 57, it was observed that in both complexes (with GCP and SCP), the copigment epicatechin has its planar surfaces (AC and B) in an optimized position to establish a direct π−π stacking and strong van der Waals contacts with CAD and B pigment planes. In both GCP and SCP complexes with epigallocatechin, the benzopyrylium provides a huge roughly planar polarizable surface that is strategically positioned in the middle of the pigment structure, establishing an accessible face and a closer contact with the B and E planes of both pigments. These facts contribute to a great association of epicatechin and epigallocatechin molecules to GCP/SCP pigments and thus to the also higher K values obtained for their complexes. For GCP/SCP-B3, it was observed that the AC' nucleus of the copigment is perpendicularly located to the CAD plane of both pigments, which hampers the establishment of nonpolar contacts between both molecules. In relation to GCP/SCP-chlorogenic acid and GCP/SCP-catechin complexes, it was noticed that there are several aromatic planes of these copigments facing to solvent molecules, which prevents a simultaneous interaction of both moieties (A and B in chlorogenic acid; AC and B in catechin) with the CAD, B and E nucleus of each pigment. Subsequently, since the

hydrophobic contacts with the aromatic nucleus of oaklins are the major contributions to the copigmentation driving force, it is expected that chlorogenic acid and catechin have the smallest copigmentation binding contants. All this structural data shows that epicatechin and epigallocatechin copigments are closer to GCP and SCP pigments than B3, chlorogenic acid and catechin molecules, which is in accordance with the first two copigments having the highest copigmentation binding constants for both pigments.

Furthermore, the copigmentation process could also be strengthened by H-bonds involving the numerous hydroxyl groups from phenolic units. Table 18 shows the most important hydrogen-bond (H-bond) interactions established within each copigmentation complex studied. As seen, with the exception of catechin, all copigments make one important H-bond with each pigment, which contribute to the copigmentation complex formation.

Table 18. Most important hydrogen bonds established between both pigments (GCP and SCP) and several copigments during the MD simulation of each complex.

Interaction	Hbond	% of occurrence
OH-E ring (GCP) - OH-AC nucleus (Epicatechin)	2.8 ± 0.1	5.8
OH-E ring (GCP) - OH-AC nucleus (Epigallocatechin)	2.7 ± 0.1	5.8
OH-CAD nucleus (GCP) - OH-AC nucleus (B3)	2.7 ± 0.1	12.7
OH-CAD nucleus (GCP) - OH-B ring (Chlorogenic acid)	2.8 ± 0.1	14.0
OH-E ring (SCP) - OH-AC nucleus (Epicatechin)	2.7 ± 0.1	5.3
OH-E ring (SCP) - OH-AC nucleus (Epigallocatechin)	2.7 ± 0.1	5.1
OH-E ring (SCP) - OH-AC nucleus (B3)	2.7 ± 0.1	22.7
OH-CAD nucleus (SCP) - OH-A ring (Chlorogenic acid)	2.7 ± 0.1	14.0

- CONCLUSION

The deoxyanthocyanidin derivatives studied (oaklins) presented intermolecular copigmentation interactions. Despite the loss of color in solution being already greatly diminished in oaklin compounds when compared to anthocyanins, not only because the pK'_a is greater but also because the mole fraction of the base is substantially higher at

higher pH values, they also demonstrated that they were capable of presenting copigmentation interactions with some wine copigments that further stabilize the flavylium cation in hydroalcoholic solutions, with higher binding constants than the ones reported for anthocyanins in some cases.

Due to their higher stability in aqueous solutions and copigment interactions, oaklins continue to be regarded as potential food colorants. Further studies are still required to test their chromatic properties at different conditions (temperature, sulfites, etc) as well as their antioxidant capabilities.

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Chapter 7

Antioxidant and antiproliferative properties of 3-deoxyanthocyanidins

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In this work, the first author was responsible for all the experimental activities with the exception of the antiproliferative studies, which were performed by Iva Fernades. He also counted with the help of Paula Araújo in the liposome preparation and oxygen consumption monitoring and with guidance from Joana Azevedo in DPPH, FRAP and liposome peroxidation inhibition assays. Luís Cruz helped and advised in the chemical synthesis of luteolinidin and apigeninidin. Guidance and advice were also obtained from the rest of the authors, regarding project planning and data interpretation.

Antioxidant and antiproliferative properties of 3-deoxyanthocyanidins

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The study of the antioxidant properties of six deoxyanthocyanidins (deoxypeonidin, deoxymalvidin, luteolinidin, apigeninidin, guaiacylcatechinpyrylium and syringylcatechinpyrylium) and an anthocyanin (cyanidin-3-glucoside) was carried out. The aim was to evaluate the relationship between the structure and the antioxidant properties of individual deoxyanthocyanidins, compared to a common derivative anthocyanin, cyanidin-3-glucoside. The ability of these compounds to inhibit lipid peroxidation in a liposome membrane system was examined by monitoring oxygen consumption and the antiradical and reducing capacities were determined using the DPPH and FRAP assay, respectively. The results showed that all the compounds tested presented antioxidant properties. Cyanidin-3-glucoside presented higher antiradical and reducing activities in the DPPH and FRAP assay, although in the liposome model, the guaiacylcatechinpyrylium was more effective inhibiting lipid peroxyl radicals.
Aditionally, the antiproliferative effects of deoxyanthocyanidins, have been evaluated against two cancer cell lines from stomach (AGS, MKN-28) and one colon cancer cell (Caco-2), and compared with the effect of their anthocyanic forms.
Overall, the compounds tested were active against caco-2 cell line, being Cyanidin-3-glucoside, Peonidin-3-glucoside and syringylcatechinpyrylium the most active.
Deoxyanthocyanidins, and in particular, guaiacylcatechinpyrylium may be regarded as potential food colourants.

KEYWORDS: deoxyanthocyanidin; pigment; antioxidants; DPPH; FRAP; liposome; food colorant

- INTRODUCTION

Deoxyanthocyanidins are considered the chemical ancestors of anthocyanins, the ubiquitous water-soluble pigments that are found in flowers and fruits and are responsible for their impressive blue and purple colors (Sweeny and Iacobucci 1977). Anthocyanins are becoming increasingly important not only as food colorants, but also as antioxidants. There are reports of therapeutic benefits including vasoprotective and anti-inflammatory properties, anti-cancer and chemoprotective properties, as well as

anti-neoplastic properties (Lietti and Forni 1976, Kamei, Kojima 1995). Anthocyanins are therefore considered to contribute significantly to the beneficial effects of consuming fruits and vegetables (Wang et al. 2012). Consequently, there has been a rising demand for natural sources of food colorants with nutraceutical benefits (Boyd 2000) and alternative sources of natural anthocyanins are becoming increasingly important (Azevedo, Fernandes 2010, Fernandes, Faria 2014).

Oppositely to anthocyanins, 3-deoxyanthocyanidins pigments do possess neither a glucose group nor a hydroxyl group in the C-ring 3-position of the flavylium core (Pina et al. 2012). This unique feature provides the 3-deoxyanthocyanins very different physico-chemical and biochemical properties, comparing to anthocyanins. Indeed, they are much more stable in slightly acidic solutions than anthocyanins and anthocyanidins (Iacobucci and Sweeny 1983, Dangles and Elhajji 1994, Khalil et al. 2010, Sousa et al. 2013), have greater resistance to colour bleaching by sulfur dioxide (Ojwang 2007) and were also recently demonstrated to be more cytotoxic to cancer cells than their anthocyanidin analogs (Shih et al. 2007), which points to the potential advantage of this type of compounds as viable commercial food colorants and justifies the research developed in the chemistry of 3-deoxyanthocyanins.

Despite their interesting color features and health benefits, the antioxidant properties of these anthocyanidin derivatives have only been briefly studied, mostly in plant extracts (Awika et al. 2005, Kayode, Nout 2011, Cardoso, Montini 2014). Only recently attempts have been made to analyze the antioxidant properties of pure 3-deoxyanthocyanidin compounds (Carbonneau, Cisse 2014).

Most of the methods used to analyze anthocyanins' antioxidant properties focus on the different mechanisms of the antioxidant defense system, such as scavenging of oxygen and hydroxyl radicals, reductions of lipid peroxyl radicals, inhibition of lipid peroxidation or chelation of metal ions (Halliwell, Aeschbach 1995, Halliwell and Whiteman 2004). On the other hand, the use of LDL or liposomes has become a more promising method for the assessment of antioxidant properties relevant to human nutrition (Storm and Crommelin 1998), since they allow the study of the protection of a specific substrate by an antioxidant in a model biological membrane or lipoprotein.

The aim of this work was thus to screen several 3-deoxyanthocyanidins for their antioxidant properties, comparing the results with a common anthocyanin (cyanidin-3-glucoside). DPPH and FRAP assays were performed to evaluate their antiradical and reducing properties, respectively. Furthermore, the activity against lipid peroxidation was

also tested using soybean phosphatidylcholine liposomes as a membrane model system. The extension of membrane lipid oxidation was followed by measuring the oxygen consumption. Additionally, the antiproliferative properties of several 3-deoxyanthocyanidins was also evaluated against two cancer cell lines from stomach (AGS, MKN-28) and one colon cancer cell (Caco-2), and compared with the effect of their anthocyanic forms.

- MATERIALS AND METHODS

Reagents. 2-cloro-3,4-dihydroxyacetophenone, 2-bromo-4-hydroxyacetophenone, 2,4,6-trihydroxybenzaldehyde, hexafluorophosphoric acid (65% in water), zinc powder, acetic acid, (+)-catechin, coniferaldehyde and sinapaldehyde, DPPH, AAPH, $FeCl_3$, DMSO, Hepes, Trolox, soybean L-α-phosphatidylcholine and NaCl were purchased from Sigma–Aldrich (Madrid, Spain). 2,4,6-Tripyridyl-s-triazine (TPTZ) and phloroglucinol was purchased from Fluka (Madrid, Spain).

Synthesis of deoxyanthocyanidins. Luteolinidin and apigeninidin were obtained through the chemical synthesis between 2,4,6-trihydroxybenzaldehyde and 3,4-dihydroxyacetophenone and 4-hydroxyacetophenone, respectively, in acetic acid in the presence of aqueous hexafluorophosphoric acid according to the procedure described elsewhere (Kueny-Stotz et al. 2007, Mora-Soumille, Al Bittar 2013).
The synthesis of the compounds 3-deoxypeonidin and 3-deoxymalvidin was followed according to the procedure described in the literature (Sousa et al. 2012).
The pigments guaiacylcatechin-pyrylium (GCP) and syringylcatechin-pyrylium (SCP) were obtained through the chemical synthesis between catechin and coniferaldehyde or sinapaldehyde, respectively, according to the procedure described elsewhere (Sousa et al. 2013).

Cyanidin-3-glucoside. Cyanidin-3-O-glucoside (Cy-glc) was extracted and purified in the laboratory from blackberries (*Rubus fruticosus*) by semipreparative HPLC with a C18 reversed phase column, as described elsewhere (Azevedo et al. 2010).

Radical scavenging assay (DPPH). Radical scavenging assays were performed using DPPH (2,2-diphenyl-1-picrylhydrazyl) as a free radical, according to the method described in the literature with some modifications (Bondet et al. 1997). DPPH reacted with the tested antioxidant compound reacted and decreased the absorbance measured at 515 nm, indicating the potential scavenging of the compounds. All the pigments tested absorbed at 515 nm, thus previous control assays were performed with all the

compounds in order to subtract their contribution at this wavelength. The assays were conducted in a microplate reader of 96 wells (Biotek Powerwave XS with software KC4). The scavenging reaction was carried out on the plate wells with a temperature of 25 °C. A solution of 60 μM DPPH was previously prepared in methanol. 270 μL of this solution was added in each well together with 30 μL of antioxidant. The compounds tested were at a final concentration of 10 μM. The decrease in absorbance was measured at 515 nm, at t=0 and every 10min, for 30min. For the final results, the 0–20 min reaction time range was used. Antiradical activity was expressed as μM Trolox equivalents. The antiradical activity was calculated from the equation determined from a linear regression after plotting known solutions of Trolox at different concentrations.

Ferric reducing antioxidant power (FRAP). The FRAP assay was performed following the method described in the literature (Benzie and Strain 1996) with some modifications. This method consists in the reduction of ferric tripyridyltriazine complex $[Fe(III)\text{-}(TPTZ)_2]^{3+}$ to ferrous tripyridyltriazine complex $[Fe(II)\text{-}(TPTZ)_2]^{2+}$ by an antioxidant. The resulting product has an absorption maximum at 593nm, which can be measured by spectrophotometry. Its formation will thus reflect the reductive capacity of the antioxidant. The reaction was performed in a microplate reader of 96 wells (Biotek Powerwave XS with software KC4). The reaction was carried out on the plate wells with a temperature of 37 °C. FRAP reagent (10 vol of 300 mm acetate buffer, pH 3.6 + 1 vol of 10 mm TPTZ in 40 mm HCl + 1 vol of 20 mM $FeCl_3$) was diluted to one-third with acetate buffer. 270 μL of this solution was added in each well together with 30 μL of compound. The blank assay was performed using 270 μL of FRAP reagent and 30 μL of methanol. The antioxidant compounds were dissolved in methanol and used in a final concentration of 10 μM. The absorbance at 593 nm was measured in time 0 and 4 min. The results were expressed as Trolox equivalents.

Liposome preparation. Liposomes were prepared by evaporation to dryness of L-μ-phosphatidylcholine (PC) from soybean solution in chloroform with a stream of argon; the film was then left under vacuum for 3 hours to remove all traces of the organic solvent. The resulting dried lipid film was dispersed with Hepes buffer (10 mM Hepes, 0.1 M NaCl, pH 7.4) and the resulting mixture was shaken above the phase transition temperature to produce multilamellar liposomes (MLV). Frozen and thawed MLVs were obtained by repeating the following cycle five times: freezing the vesicles in liquid nitrogen and thawing the sample in a water bath at 37 °C. Lipid suspensions were equilibrated at 37°C for 30min and extruded 10 times through polycarbonate filters of

100 nm pore size in a 10 mL stainless steel extruder to form large unilamellar vesicles (LUV) (Rodrigues, Gameiro 2001).

Oxygen consumption. The thermal degradation of the azo compound AAPH generated peroxyl radicals at a constant rate, which induced lipid peroxidation of soybean LUVs. The reaction was conducted in the presence and absence of antioxidants and followed by measuring the rate of oxygen consumption with a Clark-type oxygen electrode (Hansatech®) provided with an automatic recording apparatus. The reaction mixture contained 1255 µL of Hepes buffer, 223 µL of LUV (340 µM final concentration) and 2 µL of the antioxidant (1 mM initial concentration) tested, dissolved in methanol. This mixture was previously left in a 37 °C thermostated bath for 1 h and then introduced in a closed glass vessel, protected from light, thermostated at 37 °C, and provided with a stirrer. The reaction started after the addition of 22 µL AAPH (10 mM final concentration) (Porto et al. 2003). The induction periods of the compounds were determined graphically from the profiles of oxygen consumption, by the coordinates of the interception of tangents to the inhibited and uninhibited rates of oxidation (Faria et al. 2005). Results were expressed relatively to those obtained with Trolox.

Cell culture conditions. Two human cancer cell lines from stomach, AGS and MKN-28 and one from colon Caco-2, were grown as monolayer and maintained at 37 ºC in an atmosphere of 5% CO_2 and 90% relative humidity. For routine maintenance, cells were cultured in 25 cm^2 flasks as monolayer and maintained in RPMI 1640 AQmedia (AGS and MKN-28) containing 10% heat-inactivated FBS or Minimum Essential Medium Eagle (MEME) (Sigma, Germany), supplemented with 15% fetal calf serum (FCS) (Biowhittaker, Lonza, Belgium) and 2 mM L-alanyl-glutamine (Caco-2). In both cases 1% antibiotic/antimycotic solution (100 units mL^{-1} of penicillin, 100 µg mL^{-1} of streptomycin and 0.25 µg mL^{-1} of amphotericin B) was added to the medium. Cells were harvested by trypsinization (0.25%, w/v, trypsin-EDTA4-Na) twice a week.

Sulforhodamine B assay. The effect of the compounds on the growth of the human adenocarcinoma cell lines was evaluated according to the procedure adopted by the National Cancer Institute (NCI, USA) that uses the protein-binding dye sulforhodamine B (SRB) (Sigma ChemicalCo, Saint Louis, MO, USA) to assess cell growth. AGS, MKN-28 and Caco-2 cells ($1.5x10^5$ cells mL^{-1}) were spread into 96-well plates and allowed to grow for 24 h before treatment. The effect of the vehicle solvent (DMSO) was evaluated in all experiments by exposing untreated control cells to the maximum concentration (0.1%) of DMSO used in each assay. A stock solution of each of the studied compounds was prepared in DMSO and kept at -20 ºC. Appropriate dilutions

of each compound were freshly prepared just prior to every assay. Thereafter, cells were incubated with the respective polyphenol for 2 days, with a maximal solvent concentration of 0.1% DMSO. Incubation was stopped by addition of TCA (50%). After 1 h at 4 ºC, plates were washed four times with water. The plates were dried at room temperature overnight or for 1 h at 37 ºC and then were stained with a 0.4% solution of SRB for 30 min in the dark. The excess of staining solution was washed out with 1% acetic acid. The dye was eluted with tris-buffer (10 mM, pH 10.5) and quantified photometrically at 492 nm. Anti-proliferative activity was determined as percent survival, calculated by the number of treated (T) over control (C) cells x 100 (% T/C).

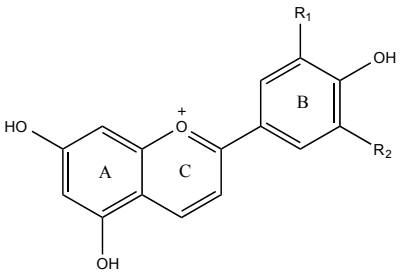
Statistical analysis. All tests were run at least in quadruplicate. Values are expressed as the arithmetic means ± standard deviation. Statistical significance of the difference between various groups was evaluated by one-way analysis of variance (ANOVA), followed by the Bonferroni test, using GraphPad Prism 5 software, version 5.01, GraphPad Software, Inc. Differences were considered significant when $p < 0.05$.

- RESULTS AND DISCUSSION

The structural differences between the deoxyanthocyanidins deoxypeonidin (Dop), deoxymalvidin (Dom), luteolinidin (Lut) and apigeninidin (Api) are due to the hydroxylation and methoxylation pattern of ring B. Oaklins, a particular case of deoxyanthocyanidins, which arise from the reaction between catechin and oak wood cinnamic aldehydes (sinapaldehyde and coniferaldehyde), present a catechin-pyrylium structure and differ between each other in the methoxylation pattern of ring B. Finally, cyanidin-3-O-glucoside (Cy-glc) is the analog of luteolinidin and has a glucose moiety linked to the OH group of ring C (Fig. 58).

The antioxidant properties of all the compounds were studied using three different *in vitro* techniques: DPPH assay, FRAP assay and monitoring oxygen consumption during lipid peroxidation of soybean PC liposomes. In addition to the tested pigments, the antioxidant activity of Trolox (water-soluble analogue of Vitamin E) was measured, so the results could be expressed in terms of ratio between the tested pigments and Trolox.

Deoxyanthocyanidins



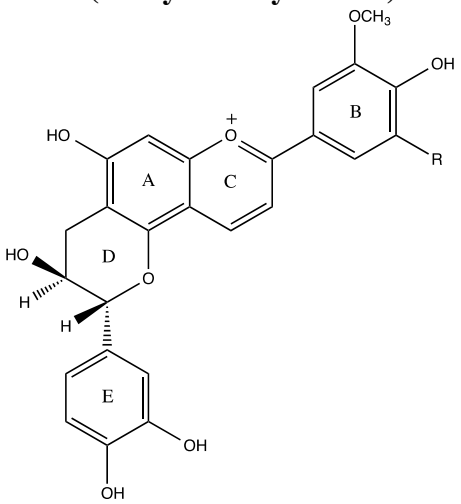
Dop: R1 = OCH_3; R2 = H

Dom: R1 = OCH_3; R2 = OCH_3

Lut: R1 = OH; R2 = H

Api: R1 = O; R2 = H

Oaklins (deoxyanthocyanidins)



Gcp: R = H

Scp: R = OCH_3

Cyanidin-3-glucoside (Cy-glc)

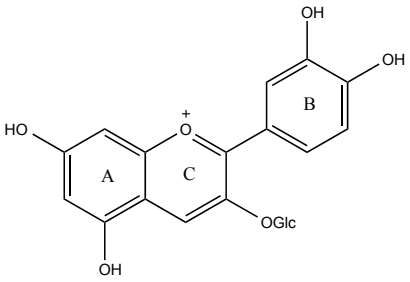


Fig. 58 - Structures of deoxyanthocyanidins: deoxypeonidin (Dop), deoxymalvidin (Dom), luteolinidin (Lut), apigeninidin (Api), guaiacylcatechinpyrylium (Gcp) and syringylcatechinpyrylium (Scp); and the anthocyanin: cyaniding-3-glucoside (Cy-glc).

DPPH. In general, the radical scavenging activity of the pigments increased with the number of hydroxyl and methoxyl groups present in the structure of the pigment. Regarding the structurally simpler deoxyanthocyanidins (Dop, Dom, Api and Lut) the results revealed that the antiradical capacity increased in the following order: Api, Dop, Lut, Dom (as shown in Fig. 59). When comparing Api with Lut, the additional hydroxyl group in ortho position in ring B may account for the higher antiradical capacity. Flavonoid structures that bear ortho-dihydroxyl groups have already been reported to

display higher antiradical activity (RiceEvans, Miller 1996). A similar explanation can be proposed to compare the results between Dop and Dom due to the additional methoxyl group.

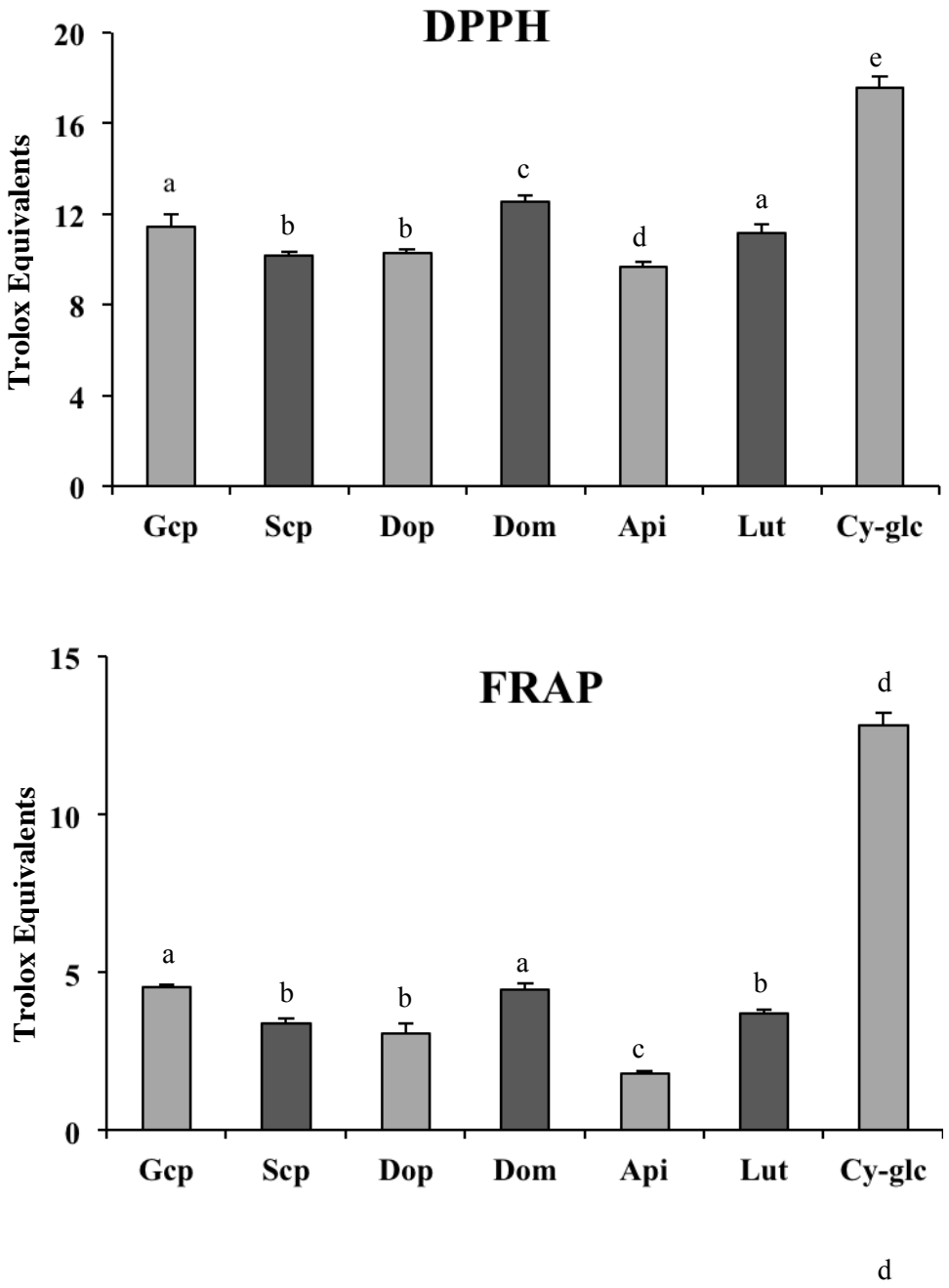


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Fig. 59 - Radical scavenging activity (DPPH) and reducing capacity (FRAP) of 10 μM deoxypeonidin, deoxymalvidin, luteolinidin, apigeninidin, oaklins (guaiacylcatechinpyrylium and syringylcatechinpyryrlium) and cyanidin-3-glucoside (μM Trolox equivalents). Columns represent mean ± standard deviation (SD), *p < 0.05. Columns with the same letter do not differ statistically.

In the case of oaklins, this trend was not observed. Instead, Scp, which features an extra methoxyl group compared to Gcp, showed a lower antiradical capacity. Nevertheless, it is interesting to note that oaklins have a higher antiradical capacity than Api, although one of them (Gcp) does not statistically differ from Lut, which like oaklins features a catechol group in its structure.

Looking into Cy-glc, the results showed a clear higher antiradicalar capacity for this anthocyanin when comparing to the tested deoxyanthocyanidins. This can be explained by the additional presence of OH groups in the glycosidic moiety (Wang, Cao 1997).

In general, the DPPH assay results obtained for Cy-glc and deoxyanthocyanidins were in accordance with previous observation on the effects of hydroxylation and methoxylation in ring B of these types of compounds to their radical scavenging ability in aqueous phase (RiceEvans et al. 1996, Wang et al. 1997, Kahkonen and Heinonen 2003).

FRAP. The results obtained using the FRAP method in order to assess the reducing capacity of the tested pigments followed a similar trend of the DPPH assay (Fig. 59). Once again, in general, the reducing capacity increased with the number of hydroxyl and methoxyl groups in the structure of the compounds.

These results are in agreement with previous studies reported for anthocyanins and derivatives (Jordheim, Aaby 2007, Azevedo et al. 2010).

Oxidation of soybean PC liposomes. The analysis of the antioxidant capacity of the pigments against oxidation of soybean PC liposomes was performed using AAPH as a peroxidation initiator. The antioxidant/antiradical capacity of the pigments was assessed at the initial and propagation stages of oxidation through the measurement of oxygen consumption, as previously described (Faria et al. 2005, Azevedo et al. 2010). The generation of peroxyl radicals from AAPH induces oxidation by hydrogen subtraction of polyunsaturated acyl chain of the liposomes, resulting in the lipid radicals that carry on the propagation phase (Esterbauer, Striegl 1989). The data obtained from the oxygen consumption assays showed that all deoxyanthocyanidins as well as Cy-glc efficiently scavenged the peroxyl radicals generated in the aqueous phase (compared to the control with no compounds) (Fig. 60).

The results were expressed in terms of ratio between the tested pigments and Trolox and there were no significant differences between almost all the compounds and also between Trolox (the values of the ratio between the compounds and Trolox were close to 1). However, we observed a higher antioxidant capacity for Gcp, even higher than

cyanidin-3-glucoside. This may be due to a higher hydrophobicity of this compound, increasing its affinity for the liposome surface and inhibiting the chain propagation of liposome-derived peroxyl radicals. The fact that the analogous compound Scp did not display significant differences from the other compounds may be due to its lower stability in aqueous solution resulting in its early degradation, which was already observed in previous studies for its thermodynamic and kinetic properties in aqueous solutions (Sousa et al. 2013).

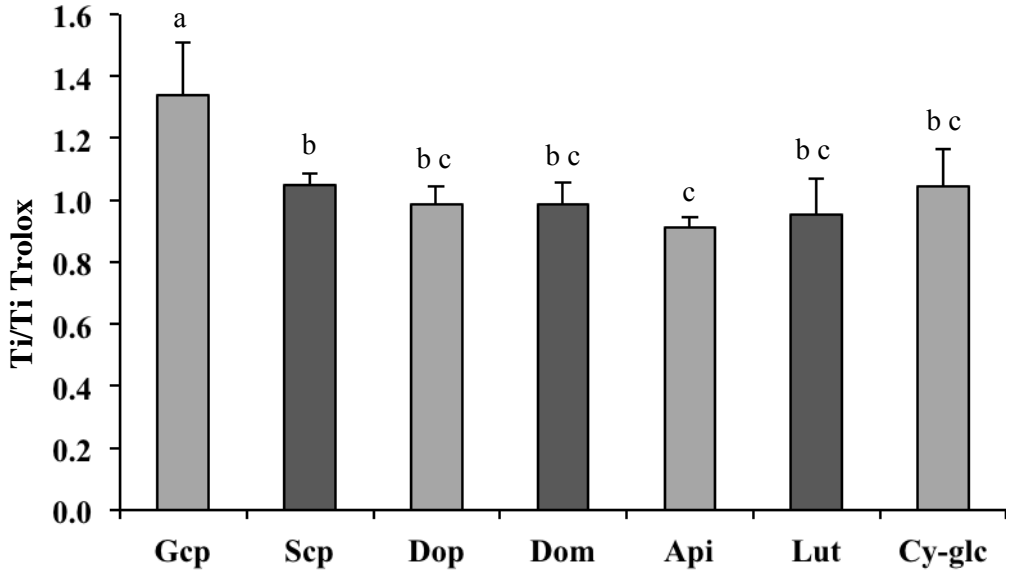

Fig. 60 - Inhibition of AAPH-initiated oxidation in soybean PC liposome by 1.25 μM of cyanidin and malvidin-derived pigments measured by oxygen consumption. Results are inhibition times in ratio to Trolox. Columns represent mean ± standard deviations (SD), *p < 0.05. Columns with the same letter do not differ statistically.

SRB. The anti-proliferative effect of deoxyanthocyanidins was studied in three cancer cell lines and compared with the effects of their anthocyanic forms (Fig. 61).

For this assay a range of 5 concentrations [6.3-100 μM] of all compounds were tested during a 48 h incubation time. To facilitate the comparison between the two groups of compounds, only one concentration was presented, 100 μM. This concentration is obviously hugely unphysiological for the colon cancer cells. However, the main goal was to make all determination in the same conditions.

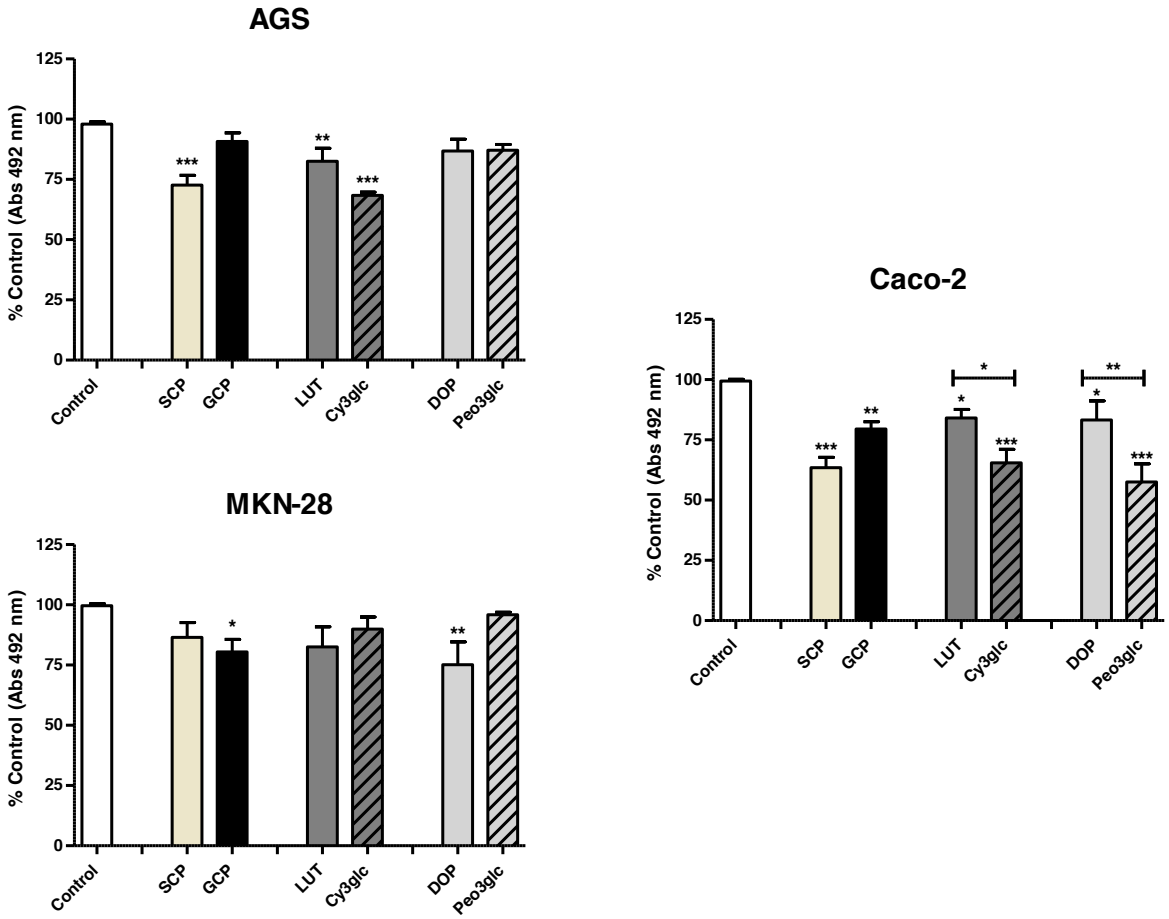

Fig. 61 - Percentage of cellular growth of AGS, MKN-28 and Caco-2 treated with different desoxyanthocyanidins and anthocyanins compared to untreated control. Cells were seeded in 96 well plates and treated with each compound (100 μM) for 48 h. Cell proliferation was evaluated by SRB assay. Each value represents the mean ± SEM (n = 6-12). *p < 0.05, **p <0.01, ***p < 0.001 (significant decrease vs control).

Also, the transient passage of compounds through stomach and intestine makes a 48h exposure a little extreme, although the 48 h time is the minimal required ensuring the duplication of the initial cells plated. This caution will allow the compounds to compromise the cellular division pathway that is one of the main mechanisms of anti-carcinogenic activity.

The effects of the assayed compounds on the growth of cancer cell lines AGS, MKN-28 and Caco-2 are shown in Fig. 61.

All compounds were active against Caco-2 cell line. The highest activity in this cell line was found for the two anthocyanins Cy3glc (65.47 % ± 5.61) and Pn3glc (57.60 % ±

7.45), and Scp (63.53 % ± 4.22), followed by Gcp (79.57 %± 2.98), Lut (84.07 % ± 3.60) and Dop (83.30 % ± 7.94).

The lack of the glucose and hydroxyl groups clearly reduces the antiproliferative activity, when comparing Cy3glc vs Lut and Pn3glc vs Dop.

Among the gastric cell lines, only Pn3glc was inactive against both cell lines. In these two cell lines, the compounds active for AGS were not active against MKN-28, this divergent activity observed may be related with some selectivity for each tumour type, AGS (poorly differentiated) and MKN28 (well differentiated).

It may also be observed that the presence of two equal substituents (two hydroxyl or methoxyl groups) may be important for activity against AGS, independently of the presence of glucose or hydroxyl group in C-3.

Considering MKN-28 cell line, the presence of the group 3-methoxy-catechol is associated with active compounds (Gcp and Dop), although that was not observed when glucose was present (Pn3glc)

- CONCLUSION

All deoxyanthocyanidins and cyanidin-3-glucoside were shown to present antioxidant properties by using three different *in vitro* techniques.

In the DPPH method, the higher antiradical capacity of cyanidin-3-glucoside was already anticipated due to the higher number of hydroxyl groups in its structure. The presence of a higher number of hydroxyl and methoxyl groups is likely to induce a significant radical scavenging capacity (Bors, Heller 1990, RiceEvans et al. 1996). This trend was observed for simpler deoxyanthocyanidins (Api, Lut, Dop and Dom) but not for oaklins (Gcp and Scp). This may be due to the lower stability in aqueous solution of Scp, which was already observed in previous studies. It was interesting to observe that the oaklin Gcp, which features a catechol group in ring E (Fig. 58), presented a higher antiradical capacity than the natural and simpler deoxyanthocyanidin apigeninidin.

For the FRAP assay we observed a similar trend. The results obtained showed that the reducing capacity of these pigments increased with the number of hydroxyl and methoxyl groups.

Overall, it was observed that all the deoxyanthocyanidins tested demonstrated antiradicalar and antioxidant properties. However, in both assays (DPPH and FRAP) the

cyanidin-3-glucoside demonstrated a much higher inhibitor capacity than deoxyanthocyanidins. This could be due to the higher number of hydroxyl groups in the glucose moiety (Wang et al. 1997) capable of reducing the TPTZ complex in the FRAP assay or scavenging radicals on the DPPH method. Other explanation may reside on its higher solubility in aqueous or hydroalcoholic solutions. Furthermore, all these compounds present several conformations in hydroalcoholic solutions and their molar fraction is dependent on several factors, which include pH, temperature and equilibration times (Sousa et al. 2013). This structural complexity makes these compounds and their antioxidant properties difficult to understand and sometimes minimal differences in experiment conditions may lead to different results.

In the oxygen consumption assays, it was observed that all compounds tested trapped AAPH-derived peroxyl radicals inhibiting the initiation of lipid peroxidation. However, the capacity demonstrated did not differ statistically amongst almost all of the pigments tested and also between the Trolox compound. However, Gcp had a higher antioxidant capacity, even higher than cyanidin-3-glucoside. This may be due to a higher hydrophobicity of this compound, increasing its affinity for the liposome surface and inhibiting the chain propagation of liposome-derived peroxyl radicals, an explanation that can be corroborated by previous studies (Azevedo et al. 2010). This latter feature may be crucial for a strategic location of the Gcp compound in the liposome surface vs. water phase, thereby influencing its effect towards protection from oxidation, as it may quench liposome-derived peroxyl radicals and thus inhibit the chain propagation, at least until exhaustion of the compound.

When considering antioxidant properties, it is important that the compound in study may apply its effect in low concentration relatively to the substrate to be oxidized and that the antioxidant remains stable after exerting its effect (e.g. the resulting radical must be stable) (Halliwell 1990, Shahidi et al. 1992). The structure of oaklins is likely to increase the overall antioxidant capacity as a result of the extended conjugation of π electrons which could stabilize the radical scavenged throughout the whole structure.

As previously mentioned, the use of liposomes constitutes a more interesting method for assessing antioxidant properties relevant to human nutrition, since it uses a model biological membrane. Thus, it was very interesting to observe that the oaklin Gcp, despite showing lower antioxidant/antiradicalar capacity in DPPH and FRAP assays when compared to cyanidin-3-glucoside, revealed a surprising higher antioxidant effect in the liposomes model.

Oaklins, which were previously shown to have interesting colour features for its use as food colourants (Sousa et al. 2013), due to their higher colour stability in acidic/neutral pH solutions, also revealed antioxidant properties similar to common anthocyanins.

Considering the antiproliferative activity all compounds were active against Caco-2 cell line, being the ones with glucose moiety and oaklin Scp the most active. A structural activity connection may be associated with the activity observed for gastric cancer cell lines since only equal di-substituted compounds were active against AGS cell line. Generally, the low percentages of inhibition observed may be related to the lack of pyrogallol group that has already been reported to be essential for antiproliferative activity of anthocyanins and other polyphenols against other cell lines (Fernandes, Faria 2010).

Regarding future perspectives, we are now in a position where further studies concerning the bioavailability of these types of compounds in *in vivo* model systems would contribute to a deeper understanding and precise knowledge of their applicability for commercial food usage.

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Conclusion and Final Considerations

The research tasks undertaken in this doctoral project had as an intent the implementation of various specific objectives initially described. In the meantime new challenges have arisen, from the dialogue with other researchers, counselors, referees from various journals and institutions and as a natural repercussion of science evolution and constant updates.

The first work of this investigation project served as a starting point for the study of new pigments with interesting color features that could arise from reactions occurring in food processing, particularly in wines. It was previously demonstrated that oaklin monomers formed from the reaction between catechin and cinnamic aldehydes have already been detected in wine model solutions (11-guaiacylcatechinpyrylium) and wine samples. The authors wanted to know if dimeric procyanidins, which are also present in real wines, could react with cinnamic aldehydes which are extracted from wood barrels during wine aging and form new pigments with diferent color properties. It was confirmed and observed the formation of orange pigments that were oaklin-catechin adducts. The hypothetic mechanism of formation of these new pigments was elucidated and the adduct formed was structurally characterized by mass spectrometry and NMR. Therefore, the authors concluded that attending to the relatively high amount of procyanidin dimers in real wines, it was also expected that oaklin–catechin adducts may also occur in wine and even play a role in some color changes observed during the aging process.

On the second work, the PhD candidate took a step further and performed a new and simple synthesis procedure to obtain 3-deoxyanthocyanidins, particularly deoxypeonidin and deoxymalvidin, from the reaction between phloroglucinol and cinnamic aldehydes (coniferaldehyde and sinapaldehyde). The resulting structures were confirmed and characterized by visible, MS, and NMR spectroscopy. Furthermore, the kinetics of formation for these compounds were studied under several conditions (pH, ethanol percentage and molar ratios). These compounds revealed themselves very useful in the lifetime of this project since they were then used for subsequent reactions to form new pigments, namely for the deoxyvitisin compounds and as model compounds for the study of the physical-chemical properties of oaklins.

In the following work, the authors were interested in studying the stability of different types of deoxyanthocyanidins, comparing their behavior and physical-chemical

properties under different pH conditions and light stimulus with analogous compounds such as common anthocyanins and other flavyliums. On this project, it was used a model compound that was synthesized in the previous work (deoxypeonidin) and also synthesized two oaklin monomer compounds, guaiacylcatechinpyrylium and syringylcatechinpyrylium.

The rate and equilibrium constants of the respective pH dependent network of chemical reactions presented by these compounds were calculated. In contrast to anthocyanins, they possessed a small *cis*−*trans* isomerization barrier and hence the rate of the *trans*-chalcone formation followed a bell-shaped curve as a function of pH. The three compounds also exhibited photochromism obtained by irradiation of the *trans*-chalcone, which, depending on pH, leads to the colored species flavylium cation and quinoidal base. When the flavylium derived networks of chemical reactions exhibit photochemistry, flash photolysis is an excellent complementary technique to collect kinetic information on the system without changing the pH. This is particularly important for flavylium derivatives possessing a low *cis*−*trans* isomerization barrier, since hemiketal, B, and *cis*-chalcone, Cc, are transient species not detected from the pH jump studies. This was the case for the deoxyanthocyanidins studied.

The flash photolysis together with pH jumps followed by UV−vis absorption and stopped flow were very useful tools to achieve the rate and equilibrium constants of the network of chemical reactions followed by these molecules.

When compared to common wine anthocyanins in which B (incolor) is the major species at higher pH values, sometimes at the pH of the wine, the oaklin compounds had a behavior more similar to simpler deoxyanthocyanidins, in which the equilibrium involves essentially AH^+, A, and Ct and the mole fraction of the quinoidal base A is approximately 50%. Furthermore, the pK'_a values obtained for GCP and SCP (3.05 and 3.2, respectively) were higher than the ones reported for malvidin-3-glucoside (2.545 and 2.327), meaning that the flavylium cation is more stable in the former. The loss of color in solution is thereby greatly diminished in oaklin compounds when compared to other anthocyanins, not only because the pK'_a is greater but also because the mole fraction of the base is substantially higher at higher pH values. In consequence, oaklins which are formed through the reaction of catechin and oak wood aldehydes may play an important role in some color changes observed in wine aging and may contribute to the overall color presented by some wines. Nevertheless, it is important to note that anthocyanins like malvidin-3-glucoside are still present in wines at concentration levels far higher than

oaklins and their contribution to wine color must not be underestimated, neither the effects of copigmentation.

Despite some differences, the general behavior of guaiacylcathechinpyrylium and syringylcatechinpyrylium was quite similar to the model compound deoxypeonidin.

This work conceded a global understanding and comprehensive analysis of the physical-chemical properties of deoxyanthocyanidins and allowed for the positioning of this family of compounds in a group distinct from anthocyanins regarding color and photochromic properties.

Given the fact that the loss of color in solution of these compounds is lower than anthocyanins, they may be regarded as potential commercial food colorants and their photochromic properties might prove also useful in other fields of research, such as interactive information systems or natural dyes.

Advancing in the search for potential compounds that might have significant stability in aqueous solution, the PhD candidate took a closer look at specific anthocyanin derived compounds, pyranoanthocyanins. These pigments have an “extra” pyranic ring which makes them much more stable towards pH variations and bleaching by SO_2 in comparison to the genuine anthocyanins. On the other hand, we have previously synthesized and characterized deoxyanthocyanidins which also had an increased stability in slightly acidid solutions compared to anthocyanins. Trying to bring the best features of these two types of compounds together, the authors have successfuly synthesized deoxyvitisins, which are deoxyanthocyanidins with the “extra” pyranic ring similar to pyranoanthocyanins.

The results showed for the first time a new class of deoxyanthocyanidins that have interesting visible spectroscopic properties, representing a step forward towards the research for new pigments with significant stability, which may prove useful in several potential applications in the food, medical, cosmetic and/or technology industries.

The color stability and the network of chemical reactions occurring in aqueous solution upon pH variations for the newly synthesized deoxyvitisins of the previous work were thouroughly investigated in the following study.

It was confirmed that like pyranoanthocyanins and opposite to anthocyanins, deoxyvitisins (3-deoxypyranoanthocyanidins) do not show hydration reactions in aqueous solutions, but undergo deprotonation reactions at higher pH values, forming only neutral and ionized quinoidal bases. The conjugated double bonds among pyranic rings C and D provide a higher electronic delocalization that prevents the nucleophilic attack of water at position 2 and the subsequent formation of the hemiketal and chalcone species. Furthermore, deoxyvitisins were found to be less acidic, thus more stable, than the corresponding pyranoanthocyanin 3-O-glucosides.

With a deeper understanding of the physical-chemical properties of deoxyanthocyanidins provided by the previous works, the PhD candidate investigated the contribution that other effects might have on color features of these compounds. From the literature, it is known that the importance and contribution of the copigmentation effect cannot be underestimated in the analysis of color changes in wine solutions. Bearing this, the authors looked for evidences of copigmentation interactions between oaklins, which have been previously identified in wines, and several copgiments: catechin, epicatechin, chlorogenic acid, epigallocatechin, and procyanidin B3.

The results showed that oaklins, like common anthocyanins, also presented copigmentation interactions that further stabilize the flavylium cation in hydroalcoholic solutions. Molecular dynamics simulations were also performed to interpret the binding data, to specify the relative arrangement of the pigment and copigment molecules within the complexes, and to interpret their absorption properties in the visible range.

Despite the loss of color in solution being already greatly diminished in oaklin compounds when compared to anthocyanins, not only because the pK'_a is greater but also because the mole fraction of the base is substantially higher at higher pH values, they also demonstrated that they were capable of presenting copigmentation interactions with some wine copigments that further stabilize the flavylium cation in hydroalcoholic solutions, with higher binding constants than the ones reported for anthocyanins in some cases. Due to their higher stability in aqueous solutions and copigment interactions, oaklins remained to be regarded as potential food colorants.

In the last work, the study of the antioxidant properties of six deoxyanthocyanidins (deoxypeonidin, deoxymalvidin, luteolinidin, apigeninidin, guaiacylcatechinpyrylium and

syringylcatechinpyrylium) and an anthocyanin (cyanidin-3-glucoside) was carried out. The aim was to evaluate the relationship between the structure and the antioxidant properties of individual deoxyanthocyanidins, compared to a common derivative anthocyanin, cyanidin-3-glucoside. The ability of these compounds to inhibit lipid peroxidation in a liposome membrane system was examined by monitoring oxygen consumption and the antiradicalar and reducing capacities were determined using the DPPH and FRAP assay, respectively. Aditionally, the antiproliferative effects of deoxyanthocyanidins, have been evaluated against two cancer cell lines from stomach (AGS, MKN-28) and one colon cancer cell (Caco-2), and compared with the effect of their anthocyanic forms.

Overall, it was observed that all the deoxyanthocyanidins tested demonstrated antiradicalar and antioxidant properties. However, in both assays (DPPH and FRAP) the cyanidin-3-glucoside demonstrated a much higher inhibitor capacity than deoxyanthocyanidins. This could be due to the higher number of hydroxyl groups in the glucose moiety capable of reducing the TPTZ complex in the FRAP assay or scavenging radicals on the DPPH method. Furthermore, all these compounds present several conformations in hydroalcoholic solutions and their molar fraction is dependent on several factors, which include pH, temperature and equilibration times. Nevertheless, in the oxygen consumption assay, Gcp had a higher antioxidant capacity, even higher than cyanidin-3-glucoside. This may be due to a higher hydrophobicity of this compound, increasing its affinity for the liposome surface and inhibiting the chain propagation of liposome-derived peroxyl radicals. This latter feature may be crucial for a strategic location of the Gcp compound in the liposome surface vs. water phase, thereby influencing its effect towards protection from oxidation, as it may quench liposome-derived peroxyl radicals and thus inhibit the chain propagation, at least until exhaustion of the compound. The use of liposomes constitutes a more interesting method for assessing antioxidant properties relevant to human nutrition, since it uses a model biological membrane. Thus, it was very interesting to observe that the oaklin Gcp, despite showing lower antioxidant/antiradicalar capacity in DPPH and FRAP assays when compared to cyanidin-3-glucoside, revealed a surprising higher antioxidant effect in the liposomes model.

Oaklins, which were previously shown to have interesting colour features for its use as food colourants, due to their higher colour stability in acidic/neutral pH solutions, also revealed antioxidant properties similar to common anthocyanins.

Considering the antiproliferative activity all compounds were active against Caco-2 cell line, being the ones with glucose moiety and oaklin Scp the most active.

This research has allowed a significative scientific advance in the study of a specific anthocyanidin derived type of coumpounds, named deoxyanthocyanidins. They have been previously identified and described but there has never been a profound and extensive study about their characteristics. This project has contributed to a broader knowledge of the physical-chemical properties of deoxyanthocyanidins and hinted at some use cases and potential applications for this type of compounds in several industries.

However, further studies are still required to test and validate their pratical use. Examples of studies that would be interesting to pursue are temperature and sulfites stress tests, as well as bioavailabilty essays. On the other hand, there is still a wide range of possibilities in the search for new deoxyanthocyanidin derived pigments with an increased stability and interesting color and photochromic properties.

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